Reprogramming of Human PBMCs into iPSCs for the In-vitro Manufacture of Engineered Vascular Grafts Based on Biodegradable Synthetic Polymers

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Melanie Generali

aus

Deutschland

Promotionskommission

Prof. Dr. François Verrey (Vorsitz)

Prof. Dr. Arnold von Eckardstein

PD Dr. Dr. Benedikt Weber (Leitung der Dissertation)

PD Dr. Paolo Cinelli (Leitung der Dissertation)

Prof. Dr. Dr. Simon P. Hoerstrup

Prof. Dr. Benjamin Gantenbein

Zürich, 2017

To my mother

Words cannot express my gratitude for everything you have done.

"The difference between stupidity and genius is that genius has it`s limits."

- Anonymous -

Table of content

Summary

Cardiovascular diseases (CVD) remains to be the leading cause of morbidity and mortality worldwide. Replacement of affected vascular tissues has been widely used to treat CVD such as coronary heart disease, aortic aneurysm and peripheral vascular disease. However, successful treatment of CVD is often limited by the lack of suitable autologous replacement tissue. Therefore, tissue engineering (TE) represents a promising solution to replace diseased vessels. TE aims at the development of constructs that integrate with the patient's native tissue to restore physiologic function. The success of any TE approach is dependent on three main factors: (i) the cell source, (ii) the scaffold matrix, and (iii) the ambient biochemical and physical factors. During the last years several different starter materials and cell sources have been investigated.

On the one hand, biodegradable scaffold matrixes form the basis of any *in vitro* tissue engineering approach by acting as a temporary matrix for cell proliferation and extracellular matrix deposition until the scaffold is replaced by neo-tissue. The present study systematically compares three frequently used polymers for the *in vitro* engineering of extracellular matrix based on poly-glycolic acid (PGA) under static as well as dynamic conditions. Ultra-structural analysis was used to examine the polymer structure. For tissue engineering (TE) three human fibroblast cell lines were seeded on either PGA-poly-4-hydroxybutyrate (P4HB), PGA-poly-lactic acid (PLA) or PGA-poly–caprolactone (PCL) patches. Later, these patches were analyzed qualitatively and quantitatively. We found that PGA-P4HB and PGA-PLA scaffolds enhance tissue formation significantly higher than PGA-PCL scaffolds. Polymer remnants were visualized by polarization microscopy. In addition, biomechanical properties of the tissue engineered patches were determined in comparison to native tissue. This study may allow future studies to specifically select certain polymer starter matrices aiming at specific tissue properties of the bioengineered constructs in vitro.

On the other hand, an ideal cell source for human therapeutic and disease modeling applications should be easily accessible and possess unlimited differentiation and expansion potential. Human induced pluripotent stem cells (hiPSCs) derived from peripheral blood mononuclear cells (PBMCs) represent a promising source given their ease of harvest combined with their pluripotent nature.

Therefore, hiPSCs were generated based on PBMCs and differentiated into smooth muscle cells (SMCs) as well as endothelial cells (ECs). These cells were seeded onto PGA-P4HB starter matrices and cultured under static or dynamic conditions to induce tissue formation *in vitro*. Resulting tissue-engineered vascular grafts (TEVGs) showed abundant amounts of extracellular matrix, containing an αSMA-positive layer in the interstitium and a thin luminal layer of vWF-positive cells approximating native vessels. These results pave the way for developing autologous PBMC-derived hiPSC-based vascular constructs for therapeutic applications or disease modelling.

Zusammenfassung

Herzkreislauferkrankungen zählen zu den häufigsten Krankheiten und sind die Todesursache Nummer 1 weltweit. In vielen Fällen ist eine Bypass-Operation unumgänglich, wie zum Beispiel bei koronare Herzerkrankungen, Aortenaneurysma oder peripheren Gefässkrankheiten. Da oftmals die körpereigenen Blutgefässe des Patienten ungeeignet sind, ist diese Behandlung nicht immer möglich. Tissue engineering (TE) stellt daher eine vielversprechende Lösung dar. Ziel des TEs ist es ein künstlich erzeugtes Ersatzkonstrukt zu produzieren, welches im Patienten die physiologische Funktion ersetzt.

Der Erfolg vom TE hängt von 3 Hauptfaktoren ab: (i) der Zellquelle, (ii) dem Zellträger (= Scaffold) und (iii) den biochemischen und physikalischen Umgebungsfaktoren. Während der letzten Jahre wurde viele verschiedene Materialien und Zelltypen getestet.

Das abbaubare Scaffold fungiert als Gerüst für den Zellwachstum und der Gewebeneubildung. Die vorliegende Studie vergleicht systematisch 3 häufig verwendete Polymere in Kombination mit einer Polyglykolsäure (PGA) unter statischen und dynamischen Bedingungen. Zur Visualisierung der Polymerstruktur wurde eine Ultrastrukturanalyse durchgeführt. Für die Gewebezüchtung wurden 3 verschiedene Fibroblastenzelllinien auf entweder PGA-Polyhydroxybuttersäure (P4HB), PGA-Polymilchsäuren (PLA) oder PGA-Polycaprolacton (PCL) kultiviert. Anschliessend wurden die Gewebekonstrukte, sogenannte Patches, qualitativ und quantitativ analysiert. PGA-P4HB und PGA-PLA stimulierte signifikant mehr die Gewebeneubildung als PGA-PCL. Polymer-Relikte wurden mittels Polarisationsmikroskopie dargestellt. Die biomechanischen Eigenschaften der gezüchteten Patches wurden im Vergleich zu nativen Gewebe gemessen. Diese Studie hilft eine ideale Auswahl eines geeigneten Scaffolds mit spezifischen Eigenschaften zu treffen.

Eine ideale Zellquelle für humane therapeutische Anwendungen und der Entwicklung eines Krankheitsmodels, sollte leicht zu gewinnen sein, und zudem unbegrenztes Differenzierungs- und Wachstumspotenzial aufweisen. Humane induzierte pluripotente Stammzellen (iPSCs) gewonnen aus peripheren mononukleären Blutzellen (PBMCs) stellen aufgrund der leichten Verfügbarkeit und dem pluripotentem Charakter eine vielversprechende Ressource dar. Aus diesem Grund haben wir PBMCs in iPSCs reprogrammiert und anschliessend in glatte Muskelzellen beziehungsweise in Endothelzellen differenziert. Beide Zelltypen wurden auf einem PGA-P4HB Scaffold statisch oder dynamisch kultiviert, um die Gewebeneubildung zu stimulieren. Die entstandenen autologen Gefässprothesen wiesen eine neugebildete extrazelluläre Matrix auf. Das Interstitium besteht aus glatten Muskelzellen, des Weiteren ist eine dünne Schicht von Endothelzellen im Lumen zu erkennen - vergleichbar mit nativen Blutgefässen. Diese Resultate ebenen den Weg für die Erzeugung eines Gefässkonstruktes für therapeutische Anwendungen und der Entwicklung eines Krankheitsmodels basierend auf iPSCs von körpereigenem Blut.

List of abbreviations

adipose-derived stem cells	ASCs
Alkaline Phosphatase	AP
arteriovenous	AV
basic fibroblast growth factor	bFGF
bone marrow stromal cells	BMSCs
cardiovascular diseases	CVD
cardiovascular progenitor cells	CVPCs
embryoid bodies	EB
embryonic stem cells	ESCs
endothelial cell medium	EGM-2
endothelial cells	ECs
endothelial nitric oxide synthase	eNOS
extracellular matrix	ECM
Food and Drug Administration	FDA
glycosaminoglycan	GAG
hematoxylin and eosin	H&E
human induced pluripotent stem cells	hiPSCs
human telomerase reverse transcriptase subunit	hTERT
hyaluronanic acid	HA
hydroxyproline	HYP
inner cell mass	ICM
knockout serum replacement	KSR
Masson Trichrome	MT
mesenchymal stem cells	MSCs
mouse embryonic fibroblast	MEF
nitric oxide	NO
peripheral blood mononuclear cells	PBMCs
poly(methyl methacrylate	PMMA
poly-3-hydroxybutyrate	PHB
poly-3-hydroxyoctanoate	PHO
poly-3-hydroxyvalerate	PHBV
poly-4-hydroxybutyrate	P4HB
poly–caprolactone	PCL
poly-D-lactideD	PDLA
polyethylene terephthalate	PET
poly-glycolic acid	PGA
polyhydroxyalcanoates	PHA
poly-lactic acid	PLA
poly-L-lactide	PLLA
polytetrafluoroethylene	ePTFE
polyurethane	PU
prostacyclin	PGI2
retinal pigment epithelial	RPE
scanning electron microscopy	SEM
servere combined immune deficiency	SCID
smooth muscle cells	SMCs
smooth Muscle Growth Medium-2	SmGM-2
smooth muscle myosin heavy chain	SMMHC
tetrahydrofuran	THF
tissue engineered heart valves	TEHVs
tissue engineered vascular grafts	TEVGs
tissue engineering	TE
tissue-type plasminogen activator	t-PA
von Willebrand factor	vWF
wet-type macular degeneration	AMD
α smooth muscle actin	αSMA

Chapter 1

Introduction

Polymeric Starter Matrices for Cardiovascular Tissue Engineering

Petra E. Dijkman*, Melanie Generali*, Benedikt Weber and Simon P. Hoerstrup

* These authors contributed equally to the study

Regenerative Medicine Program, Division of Surgical Research, University and University Hospital of Zurich, Zurich, Switzerland

Encyclopedia of Biomedical Polymers and Polymeric Biomaterials (published 2015)

1.1 Introduction

1.1.1 Cardiovascular diseases and current therapies

Cardiovascular diseases (CVDs) are the leading cause of death and disability worldwide, claiming 17.3 million lives each year and accounting for 30% of all global deaths [1]. Due to an increase of co-morbidities an ever ageing population, mortality numbers are expected to increase to about 23.3 million a year in 2030 [1]. CVDs in general cost more than any other disease and are projected to remain the single leading cause of death. The enormous annually costs related to this type of diseases has reached more than $33 billion in the US and more than £700 million in the UK [1]. Moreover, the total direct (health expenditures) and indirect (decreased productivity resulting from morbidity and mortality) costs of cardiovascular disease and stroke in the United States for 2010 is estimated at 503.2 billion dollars, from which the total hospital costs projected for the year 2010 are estimated to be 155.7 billion dollars [2].

Unfortunately, in many situations the successful treatment of vascular diseases is limited by the lack of suitable autologous tissue to serve as vascular conduits to replace or bypass diseased or occluded vessels or repair or replace dysfunctional valves. Originally, autologous vascular grafts have been used in the field of reconstructive arterial surgery to treat vascular diseases, such as atherosclerosis that lead to the sequel of myocardial infarction and stroke [3]. However, their preparation increases time, cost, and the potential for morbidity to the surgical procedure [4, 5]. Therefore bypass grafts engineered from synthetic materials, e.g. Dacron or ePTFE, have been developed and successfully used for the replacement of larger vessels, i.e. 6–10 mm in diameter [6, 7]. However, when used in the coronary system (inner diameter <6 mm) occluding of the vessels is a major problem due to thrombosis and hyperplasia.

Valvular heart disease is another important cause of morbidity and mortality and the number of patients requiring heart valve replacement is approximately 280.000 annually worldwide [8]. As the group of congenital heart disease patients is expected to increase and the number of valve replacements is estimated to triple to over 850.000 over the upcoming five decades [9], the search for alternative heart valve replacements is ongoing. Cost wise, the development of a living valve replacement, i.e. by tissue engineering technologies, could save up to 36 billion dollars per 10 years for just US healthcare by eliminating both re-operations, to replace degenerated cryopreserved homografts, and the total cost per patient lifetime after mechanical valve implantation [10]. In addition, for infants and children, the elimination of re-operations to replace non-growing valve replacements will reduce their mortality rate and suffering. However, currently available valve substitutes are either mechanical or bioprosthetic valves. Despite excellent durability, mechanical valves have a substantial risk of thromboembolic complications. Additionally, the required life-long anticoagulation therapy potentiates hemorrhagic complications. In contrast, bioprosthetic valves show similar flow patterns to that of the native heart valves and, therefore, have a low risk of thromboembolism without anticoagulation. However, their durability is limited due to calcific or noncalcific tissue deterioration [11].

A major drawback of all artificial cardiovascular replacements is their absence of repair, remodeling and growth capacity, the latter being particularly important in children and young adults. Tissue engineering is suggested as a solution to these problems by replacing tissue or organ function with engineered tissue created with the help of bioresorbable starter materials. The ultimate goal in cardiovascular tissue engineering is to develop autologous tissue engineered vascular grafts (TEVGs) and – heart valves (TEHVs) that are immunologically compatible, non-thrombogenic, and possess growth and remodeling capacity. The success of an engineered tissue replacement is based on mimicking the composition and structure of the original tissue. Therefore it is necessary to understand the specific demands of the cardiovascular system requiring an enormous strength, flexibility and durability of the engineered structures, as well as a high degree of adaptive capacity to cope with changes due to growth, physical activity and pathological conditions.

1.1.2 Principles of tissue engineering

The field of tissue-engineering applies the principles of biology and engineering to the development of biological substitutes for the repair or regeneration of tissue or organ function [12]. In 1993, Langer and Vacanti summarized the early developments in this field and defined the original tissue engineering paradigm that comprises a scaffold which is seeded with cells isolated from the recipient, subsequent in-vitro tissue formation and in-vivo tissue growth and remodeling following implantation [13]. The in-vivo phase can involve recruitment of the recipient's inflammatory cells, resulting in a combination of seeded and recipient-derived new cells in the engineered construct [14]. Both in-vitro and in-vivo, the key processes during tissue formation and maturation are cell proliferation and migration, extracellular matrix production and organization, and scaffold degradation. These capacities of the tissue engineered construct that enable repair of structural injury, remodeling of the extracellular matrix, and potential growth are crucial for long-term success of the living engineered tissues [15]. Various approaches to engineer such durable tissue constructs have been investigated and can be roughly divided into three groups, namely *in-vitro, in-vivo,* or *in-situ* tissue engineering.

In-vitro tissue engineering: The first approach aims at the development of the construct in-vitro according to the classical paradigm. After seeding (autologous) cells onto different types of scaffold material, the construct is cultured in-vitro to stimulate tissue formation.

In-vivo tissue engineering: Secondly, the in-vivo tissue engineering approach relies on autologous tissue formation by intraperitoneal implantation of a scaffold [16-20]. After sufficient autologous tissue formation in-vivo, the construct is transplanted to serve as a cardiovascular replacement. Although this is a very attractive method to create autologous tissue, the current status for this approach is that the volume fraction of cellular phenotypes is unbalanced, resulting in for example inadequate remodeling of heart valve replacements [16].

In-situ tissue engineering: The third approach, although not fully complying with the classical concept of tissue-engineering as defined by Langer and Vacanti, made the first step towards clinical implementation by implanting a scaffold into the patient relying on spontaneous endogenous cellular repopulation [21]. For this in-situ tissue-engineering approach the regenerative capacity of the body is utilized. Suitable starter materials are implanted directly, without or directly after in-vitro preseeding, in order to recruit endogenous cells in-vivo. More recently, direct implantation of smart synthetic scaffold materials is proposed that actively attract the circulating endogenous cells. It is hypothesized that the scaffold will be populated by endogenous cells and subsequently, in-vivo tissue formation will take over the function of the degrading scaffold [22].
As will be reviewed in this chapter, various scaffold materials are extensively investigated for the in-vitro and the in-situ approaches of cardiovascular tissue engineering. These synthetic or natural based materials are used to create heart valves and vessels (see also Table 1 and 2) and to a lesser extend to create cardiac muscle tissue. Additionally, various cell sources are investigated for tissue engineering.

1.1.3 Cell sources for cardiovascular tissue engineering

Despite being most important for the viable part of the construct, in contrast to the controllable properties of the starter materials, the cell source is still the least controllable factor. Their regenerative capacity and quality to form tissue relies on the characteristics of the origin and thus varies between individual donors and original organ tissue. A variety of cell types have been investigated for cardiovascular tissue engineering. For human applications, the ideal cells should be non-immunogenic, functional, and easy to isolate and expand [23]. Obviously, the cell sources mostly investigated for cardiovascular tissue engineering are the non-immunogenic autologous endothelial cells (ECs), smooth muscle cells (SMCs), and (myo) fibroblasts, as listed in Table 1 and 2. In order to obtain autologous cells, a piece of donor tissue is dissociated into individual cells, expanded in culture and finally attached to a scaffold and implanted.
During the last years stem cells have attracted more attention and have become an important cell source for tissue engineering to replace lost host cells or to induce endogenous repopulation, in particular for cardiac muscle regeneration [24, 25]. Stem cells can be found in embryos, fetuses, and in adults and by definition they have the ability to reproduce themselves for a long period of time (self-renewal) and to give rise to different cells types (differentiation). There exist different stem cells, depending on their differentiation potential: the embryonic stem cells (ESCs) and the adult stem cells. The ESCs have the ability to give rise to all types of cells that form the three germ layers (mesoderm, endoderm, and ectoderm) from which all the cells of the body arise. ESCs are derived from the pre-implantation embryo, precisely from a group of pluripotent cells called inner cell mass (ICM). Once removed from the blastocyst, the cells of the ICM can be cultured under special conditions in-vitro and are then called ESCs [26, 27]. Just few studies have described attempts to engineer ESCs derived patches for cardiac repair [28-30]. For example, Ke et al. implanted scaffolds seeded with undifferentiated mouse ESCs in mice whereby scar size and ventricular dilatation were reduced [28]. Despite initial success [30], ethical issues, immunogenic and tumorigenic problems are the

major drawbacks. The adult stem cells have a more restricted (multipotent) differentiation potential compared to ESCs. They are found in adult organisms where they can give rise to specialized cells that give rise to the different tissues of the body. Adult stem cells serve for both homeostasis in healthy tissues and for regeneration of defective tissues [31]. Most of the recent investigations were focusing on using bone marrow stromal cells (BMSCs) and adipose-derived stem cells (ASCs) for cardiovascular tissue engineering [32, 33].

Recently, another interesting cell source for regenerative medicine, the induced pluripotent stem cell (iPSC), has become available. As part of this approach differentiated, adult cells have been genetically reprogrammed to an embryonic stem–like state with pluripotent differentiation capabilities. In 2006 Takahashi and Yamanaka demonstrated the induction of pluripotent stem cells from mouse embryonic and adult fibroblasts [34]. Using genetic reprogramming with the transcription factors Oct4, Sox2, Klf4 and c-Myc, somatic cells converted into iPSCs. iPSCs are very similar in morphology, proliferation and gene expression to ESCs [35] and represent a powerful tool in regenerative medicine. Unfortunately, iPSCs have been associated the risk of teratoma formation and so more investigation is needed into the use of these cells for autologous cell based therapies such as cardiovascular tissue engineering. An open question is still whether an optimal approach would involve seeding with undifferentiated iPScs or rather using these cells to make a differentiated cell line of SMCs and/or ECs before seeding them onto scaffold matrices for cardiovascular tissue engineering [36].

1.2 Polymeric starter matrices for cardiovascular tissue engineering

The use of polymeric materials to serve as starter matrices for cardiovascular tissue engineering has been extensively investigated. It is the cyclic loading of the cardiovascular system that requires significant durability and flexibility of the tissue-engineered cardiovascular replacements and consequently appropriate mechanical properties of the starting scaffolds to endure the cyclic stresses and strains exerted after implantation. The mechanical properties of the scaffold also provide an important stimulus to the cells for extracellular matrix (ECM) production and remodeling, as the cells experience different local stresses and strains depending on the biomechanical profile of the scaffold. However, these mechanical cues - as result of the scaffold stiffness - can modulate the differentiation of cells into pathological phenotypes, e.g. osteoblastic or myofibroblastic differentiation [37]. Additionally, bioactive factors can be incorporated into the scaffold that can direct local cellular function, or promote recruitment of specific cell types via chemotaxis.

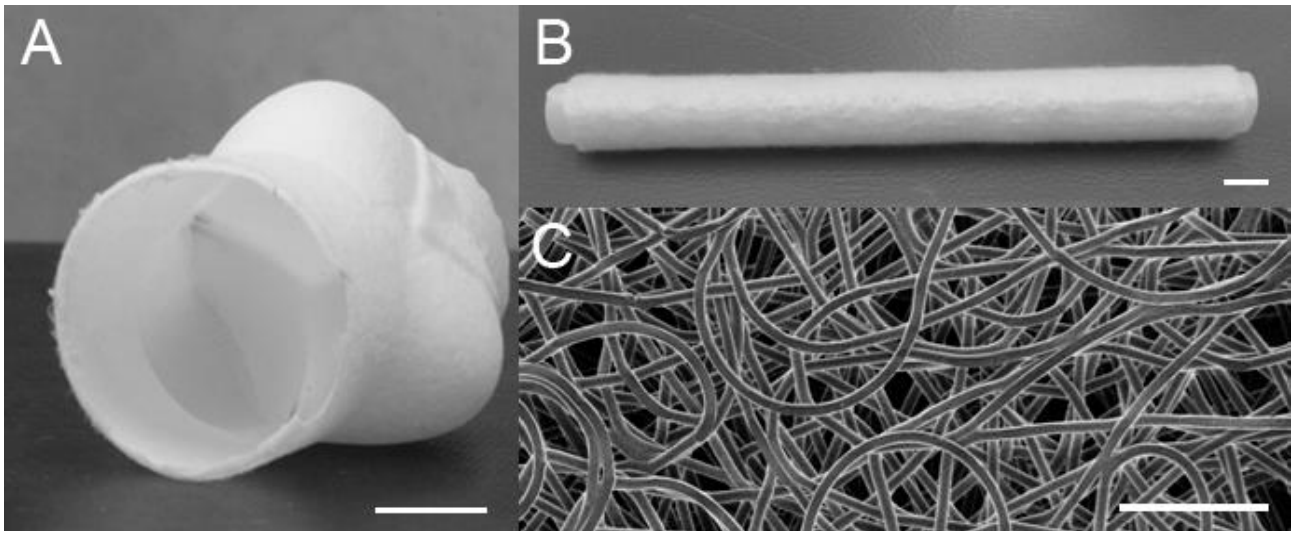

Figure 1: Example of porous scaffolds for cardiovascular tissue engineering. (A) Photograph of an electrospun poly(ε-caprolactone) heart valve demonstrating 3D valve architecture. (B) Photograph of a PGA tube for culture of a vascular graft. (C) Scanning electron micrograph of the microstructure showing the random aligned microfibers. Scale bars represent 1 cm in A and B and 100 µm in C. (Images courtesy of M. Simonet and R. Stenger.)

1.2.1 Synthetic polymers

Synthetic polymers have been extensively used in the field of tissue engineering, mainly due to their high durability and mechanical strength. Additionally, production conditions of synthetic polymers can be tightly controlled, thus, mechanical and physical properties are predictable and well defined. In addition, these scaffolds are less expensive, better reproducible and may be stored off-the-shelf over longer time periods. These characteristics make synthetic polymers to an interesting raw material for scaffold fabrication (see also Fig. 1). Nevertheless, their biocompatibility issues harbor also complications. In the following, the most important synthetic polymer materials for cardiovascular tissue engineering are presented in a summarized fashion.

1.2.1.1 Non-degradable synthetic polymers

Expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (PET) and polyurethane (PU) have been used to produce synthetic vascular grafts [38]. ePTFE is a porous polymer with an electronegative luminal surface that is nondegradable. The chemical composition supports low thrombogenicity, lower rates of restenosis and hemostasis, and less calcification [39, 40]. e-PTFE grafts have been used as vein grafts, but only 45% of standard ePTFE grafts are patent as femoropopliteal bypass grafts at 5 years, however, autologous vein grafts have 60 to 80% patency [41, 42]. In contrast, PET is less used as a graft for femoropopliteal bypass surgery but is more common used for aortic replacement. PET, or also called Dacron®, is a thermoplastic polymer manufactured by Maquet Cardiovascular Inc.. Dacron displays a good stability and can exist for more than 10 years after implantation without significant deterioration [42]. However, ePTFE and PET surfaces are thrombogenic and relatively rigid, they are not suitable for the fabrication of small diameter vascular grafts [43]. PU has been suggested as a good alternative due to it is both haemocompatible and elastic [44]. In order to improve synthetic graft function and yield biohybrid

conduits, different components have been added to the graft design. For instance, Nakawaga et al. engineered a poly(ether-urethane) graft reinforced with knitted polyester fibers for hemodialysis that was finally observed to be more durable than ePTFE [45]. Unfortunately, all three polymers have the disadvantage of being a synthetic non-degradable material and can cause a foreign body reaction with increased chances of thrombus formation. As the ideal scaffold for tissue engineering should demonstrate bioresorbable properties, the synthetic bioresorbable polymers have found extensive applications in tissue engineering.

1.2.1.2 Bioresorbable synthetic polymers

Bioresorbable synthetic polymers including poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(hydroxy alkanoate) (PHA), poly(caprolactone) (PCL) and their copolymers have generated immense interest as tissue engineering materials due to their degradable properties. The degradation of implants is of special interest for the medical industry, as no further surgical procedure is required to remove the implant. Biomaterials used in clinical applications should have degradation and resorption rates which are compatible with the healing rates of the neotissue, c.q. there must be a balance between material degradation and ECM production [46, 47]. Polymers can degrade by (1) hydrolysis, (2) oxidation ,(3) enzymatic, and (4) physical conditions [48]. Production conditions of synthetic polymers can be controlled, thus, mechanical and physical properties are predictable including degradation rates and elastic modulus. Furthermore, these scaffolds are less expensive, better reproducible and may be stored over longer time periods. These characteristics make synthetic polymers to an intensely interesting raw material for scaffold fabrication.

Because the words biodegradable and bioresorbable are often used misleadingly in literature, we will distinguishing them according to the following definitions formulated by Vert et al. [49, 50]: The term biodegradable applies to polymeric materials and solid devices that undergo dispersion as consequence of macromolecular degradation. Although degradation products can be removed from the site of action, their degradation products will not completely be eliminated and remain inside the human body. Bioresorbable materials are solid polymers and devices that degrade into non-toxic products (low–molecular weight compounds) which will be eliminated via metabolic pathways (i.e. the citric acid cycle) or directly via renal excretion without residual side effects [49, 50]. As in tissue engineering one aims to develop substitutes with the capacity to become living copies of their native counterparts without leaving remnants of the starter materials in the body, this chapter focusses on the available bioresorbable starter materials used for cardiovascular tissue engineering.

Aliphatic Polyesters have been known and studied since 1930s [51]. Their success in tissue engineering relies mainly on their degradability and biocompatibility, as well as their good processability and mechanical feature. Currently, the most widely investigated and most commonly used biomedical aliphatic polyesters are PGA, PLA, and PCL. PGA is a rigid thermoplastic material with high crystallinity, whereby it is not soluble in most organic solvents. PGA is usually synthesized by ring-opening polymerization of glycolide, the cyclic

dimer of glycolic acid [52]. Similarly, lactic acid is polymerized to synthesize PLA. Lactic acid, which is normally produced by muscular contraction, can be eliminated through the citric acid cycle whereas glycolic acid may be eliminated directly in urine or may be converted to enter citric acid via pyruvic acid [53]. Due to the chiral nature of PLA, several distinct forms of exist: poly-L-lactide (PLLA), poly-D-lactideD (PDLA) and LD racemic (PDLLA), respectively. It is known that the characteristic of PLA are highly affected by stereo-isomeric L/D ratio of lactate units. Generally, the increased stereo-isomeric ratio decreases the crystallinity, whereby the degradation is enhanced. For example, degradation of PLA is faster than for PDLA due to the lower crystallinity of PLA.

PCL is a semi-crystalline aliphatic polyester which is synthesized by ring-opening polymerization of ε-caprolactone [54, 55]. It displays good mechanical characteristics, such as high elongation and strength. PCL degrades very slowly in-vivo by enzymatic action and by hydrolysis [54]. All three polyesters are a Food and Drug Administration (FDA) approved polymers for clinical use [56].

In 1998, Shinoka et al. reported surgical implantation of TEVGs in lambs, in which scaffolds were constructed from autologous myofibroblasts and endothelial cells seeded onto PGA grafts [57]. This study demonstrated the first vascular graft using autologous cells that yielded a viable structure [57]. Despite PGA is a bioresorbable polymer, breakdown products are acidic, which could induce an inflammatory response. Furthermore, PGA degrades faster than PLA that result in a low mechanical property of TEVGs [58]. Further studies have been conducte , for instance, by using PGA-PLLA scaffolds for microvessels in mice [59] or scaffolds composed of polyglycolide knitted fiber, and an L-lactide and ε-caprolactone copolymer sponge for TEVGs in a canine inferior vena cava model [60]. The hybrid polymeric scaffold fabricated from either PGA or PLA fiber-based mesh coated with a 50:50 copolymer of L-lactide and ε-caprolactone (PCLA/PGA or PCLA/PLA) are more elastic than the PGA scaffold, resulting in an improved compliance match between the vessel and the conduit, resulting in better surgical handling characteristics [61].

Polyhydroxyalcanoates (PHA): Another group of polyesters is the PHA family that is built from hydroxyacids produced by microorganisms under unbalanced growth conditions [62]. They are generally bioresorbable and thermoprocessable and includes poly-3-hydroxybutyrate (PHB), copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), poly-4-hydroxybutyrate (P4HB), copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate (PHBHHx) and poly-3-hydroxyoctanoate (PHO) The PHA family polymers have been displayed to be convenient for tissue engineering [63]. However, a disadvantage of some PHA polymers is their limited availability and the time-consuming production by bacteria. In order to adjust mechanical features or biocompatibility PHA polymers can be either blended, surface modified or composed with other polymers, enzymes or inorganic materials [64]. Additionally, their degradation rate can be tailored by varying their copolymer ratio. For example in 1999, TEVGs based on a PGA and PHA scaffold were implanted in the abdominal aortas of lambs and successfully followed up to 5 months in-vivo. Overtime, the mechanical properties of these preseeded TEVGs changed towards those of native blood vessels [65]. For both heart

valve and vascular tissue engineering the use of PGA coated with P4HB, meaning the combination of the thermoplastic characteristic of P4HB and the high porosity of PGA, has been investigated intensively presenting promising results in in-vitro and pre-clinical studies [33, 66-70]. Hoerstrup et al. was in 2006 the first to provide the evidence of growth of living, functional pulmonary arteries engineered from vascular cells seeded on PGA/P4HB scaffolds in a growing lamb model [71]. The evidence of the growth and remodeling capacity of these implants proved their potential also for use in pediatric applications.

1.2.2 Bioresorbable natural polymers

Whereas synthetic materials have performed better in durability and strength, they pale in comparison with the functional capabilities of natural tissues. A good alternative to the synthetic polymers are natural polymers which possess biologically recognizable side groups. The category of natural-based materials for scaffolds includes polysaccharides (aliginate, chitin/chitosan, hyaluronic acid derivate), proteins (soy, collagen, fibrin, gels, and silk) or decellularized extracellular matrix (ECM). Natural polymers are used as pure materials or in combination with synthetic polymers or inorganic substances to produce scaffolds [72-74]. The natural polymers mostly used for cardiovascular tissue engineering are collagen, fibrin, and decellularized ECM.

1.2.2.1 Polysaccharides

Polysaccharides are polymeric carbohydrates consist of long chains of monosaccharide units, which are linked together by O-glycosidic bonds. Physical properties, such as solubility, gelation and surface properties can be influenced by monosaccharide composition, chain shapes and molecular weight. Polysaccharides show good hemocompatibility properties, due to chemical similarities with heparin. Furthermore, they are non-toxic, display interaction with living cells and, most of them have low costs in comparison with others biopolymers such as collagen [75]. Several polysaccharidic polymers, including chitosan, aliginate and starch have been widely proposed as scaffold materials in tissue engineering applications and will be described in more detail in the following sections.

Chitosan is the N-deacetylated derivative of chitin, a natural polysaccharide that is present in the hard exoskeletons of crustaceans and insects [76]. Chitosan is a relatively inexpensive product because chitin is one of the most common polysaccharides found in nature. Recently chitosan has started to be interesting in the tissue-engineering field due to several desirable properties, for instance, (i) the body reaction is minimal, (ii) process conditions are mild, and (iii) properties are controllable [77, 78]. In addition, chitosan is more bioactive than other degradable polymer leading to acceleration of wound healing. It has already been used for the engineering of cartilage, nerve, and liver [78]. Low strength and inconsistent behavior with seeded cells are difficulties which are associated with chitosan [79]. Nevertheless, in order to increase its strength and cell-attachment ability it can be combined with other materials such as synthetic polymers [80]. A variety

of formats including hydrogels, microcapsules, membranous films, sponges, tubes, and a variety of three-dimensional porous structure have been investigated by using chitin-based materials [81, 82]. Firstly, in 1999 Matthew et al. fabricated a family of chitosan scaffolds, including heparin-modified porous tubes, which had promising potential for application in TEVG [83]. Nevertheless, researchers developed a chitosan-based tubular scaffold which displayed proper swelling property, burst strength of almost 4000 mmHg, and high suture-retention strength not until 2006 [84].

Alginate is a linear polysaccharide derived from brown algae and is composed of repeating guluronic and manuronic units. Physical and mechanical properties of alginate are highly related to chain length. Alginate is a bioresorbable polymers that forms a solid gel under mild processing conditions, thus cells can be entrapped into beads and other shapes [85, 86]. Furthermore, encapsulation of certain cell types into alginate beads may actually improve cell survival and growth [87]. Some disadvantages of alginate have been published, including mechanical weakness and poor cell adhesion [85]. Again, to overcome these limitations, the strength and cell behavior of alginate have been enhanced by combining alginate with agarose [87] and chitosan [85]. Nevertheless, alginate has been investigated in liver [88], heart [89], and cartilage [90] tissue-engineering. For example, Leor et al. demonstrated the feasibility of bioengineering a cardiac tissue within alginate scaffolds [91]. They implanted a cardiac graft onto rat infarcted myocardium which stimulated intense neovascularization from neighboring coronaries [91].

Starch, a natural polymer, is a material that is also commonly used in tissue engineering. It is deposited in the chloroplasts of plant cells (e.g. corn, rice, and wheat) as insoluble granules composed of α-amylose (20–30%) and amylopectin (70–80%). Starch is degraded by enzymes amylases to lower molecular weight chains of fructose and maltose. Starch-based polymers have been selected as good biomaterials due to their biocompatibility and degradability [92]. Furthermore, starch is highly available, cheap and can be easily modified. In most relevant studies starch has been blended with other polymers to produce composite materials with improved properties for tissue engineering scaffolds [93, 94]. Recently, it has been demonstrated that starch-based fibers permit adhesion of endothelial cells, an indicator of the ability to permit vascularization [95]. These results confirm again the potential of these materials for tissue engineering.

Hyaluronan, also known as hyaluronanic acid (HA), is a naturally occurring non-sulfated glycosaminoglycan (GAG) and is obtained from the extracellular matrix of most connective tissues such as cartilage, vitreous of the human eye, umbilical cord and synovial fluid [96]. Hyaluronan is a linear polysaccharide composed of N-acetylglucosamine and glucuronic acid, linked by β(1→3) bonds. In the ECM of connective tissues, HA has many physiological roles, including maintaining tissue morphologic organization, structural and space-filling properties, and transporting ions, solutes, and nutrients [96]. Three types of enzymes have been found in

mammals which may participate in the enzymatic degradation of HA: (1) hyaluronidase, (2) b-D-glucuronidase, and (3) b-N-acetyl-hexosaminidase. The *in vivo* degradation time of unmodified HA varies from few minutes in blood to hours or days in skin [97, 98]. The degradation products of HA are 4–20 disaccharides in length which display angiogenic properties, stimulating capillary growth, endothelial cell proliferation and migration [99, 100]. Interestingly, HA inhibits the adhesion and aggregation of platelets, and these nonthrombogenic properties are of particular importance in cardiovascular tissue engineering [101]. HA was used for the luminal surface coating of vascular grafts [102]. Hyaluronan has been used for different tissue engineering applications [103-105], most likely due to the fact that it is a macromolecule of the ECM, thus mimics the native environment during tissue development.

1.2.2.2 Natural proteins: Collagen, Gelatin, and Fibrin

With respect to protein-based polymers, collagen, fibrin, and gelatin will be described in more detail in the following sections. Protein-based polymers have the benefit of mimicking many features of ECM and thus have the potential to control migration, growth and organization of cells during tissue regeneration process.

Collagen molecules have a triple-helical structure and provide high tensile strength due to the arrangement of triple helices in fibrils. This biopolymer is the major protein component of the ECM and plays a dominant role in maintaining the biologic and structural integrity. There are four main collagen types (I, II, III, and V) that make up the essential part of collagen in bone, cartilage, tendon, skin, and muscle [106, 107]. The most explored collagen for biomedical applications is collagen type I. Collagen scaffolds have been investigated for blood vessels, heart valves and ligaments [108]. The possible degradation by human collagenases makes collagen an ideal scaffold for tissue engineering and could potentially lead to the restoration of tissue structure and functionality [109]. The degradation rate often needs to be regulated using diverse methods such as crosslinking techniques [110]. A variety of formats including porous sponges [111], gels and sheets [112, 113], and foams [114] have been investigated by using bioresorbable collagen. The feasibility of TEVGs made of collagen and cells was first demonstrated in 1986 by Weinberg and Bell [115]. Despite the low degradation rate, collagen has the distinctive drawback that the availability of homologous (human) collagen is very low as it is difficult to obtain from patients.

Gelatin is derived from collagen and formed by breaking the natural triple-helix structure of collagen into single-strand molecules by hydrolysis. Gelatin is less immunogenic compared with its precursor. It is commonly used for pharmaceutical and medical applications due to its biodegradability and biocompatibility in physiological environments [116]. Additionally, cell adhesion, migration, differentiation and proliferation can be presumably promoted due to the existence of informational signals like the Arg–Gly–Asp (RGD) sequence [117, 118]. Gelatin blended with chitosan has been used in artificial skin and cartilage applications because of the ability to form a polyelectrolyte complex [119]. Li et al. published that bioengineered cardiac

grafts can be fabricated by fetal cells and 3D gelatin mesh [120]. Interestingly, spontaneous contraction was observed *in-vitro* as well *in-vivo*. Nevertheless, histological analysis could not confirm the identity of the cells [120].

Fibrin is a biopolymer of the monomer fibrinogen, meaning it is the end-product of the coagulation cascade following the conversion of fibrinogen in the presence of thrombin and calcium [121]. Fibrinogen is a soluble plasma glycoprotein, which is produced by the liver. Fibrin and fibrinogen play essential roles in blood clotting, fibrinolysis, cellular and matrix interactions, the inflammatory response, wound healing, and neoplasia [121]. Fibrin is used as a naturally-occurring scaffold that can be produced from the patient's own blood and used as an autologous scaffold without the potential risk of a foreign body reaction [122]. Based on their autologous and bioresorbable properties, many applications for tissue engineering has been established in combination with cells, growth factors, or drugs [123-125]. The most broadly used forms of fibrin scaffolds are fibrin hydrogels, fibrin glue, and fibrin microbeads [126]. Fibrin vascular constructs are weaker and more extensible than collagen-based constructs. Therefore mechanical properties can be improved by fibrin-collagen composites presenting higher strength than collagen alone, but also more gel compaction [127]. Interestingly, fibrin gels can stimulate SMC to synthesize elastin, which is an important component of arteries [128]. However, fibrin hydrogels display three major disadvantages: the shrinkage of the gel, low mechanical stiffness, and its rapid degradation before proper formation of tissue engineered structures [123, 129]. On the other hand, as fibrin is known to degrade within several days by cell-associated enzymatic activities, it can be utilized for the controlled release of growth factors [130]. Moreover, by adding for example aprotinin, that restricts or even stops fibrinolyse, the degradation can be controlled [129]. Aprotinin is a monomeric serine protease inhibitor found to effectively inhibit the activity of several proteases, including plasmin, trypsin, chymotrypsin, and kallikrein [131].

1.2.2.3 Decellularized Extra Cellular Matrix (ECM)

Besides using proteins, such as fibrin or collagen, also the complete natural ECM has been discovered as an appropriate matrix for tissue engineering. The ECM works as a supporting material and regulator of cellular functions including cell survival, proliferation, morphogenesis and differentiation [132]. Many organs and tissues, such as vessel, heart valves, and pericardium from humans and animals (e.g. sheep, pigs, and rabbits) have been studied for use as starter matrix [133-136] and in-vivo complete cellular ingrowth was proved in animal models [137, 138]. However, the use of either xenogenic or allogenic ECM increases the risk for immunogenic reactions. Moreover, using unfixed biological material, and in particular when using xenogenic matrices, there is a risk of disease transmissions, such as Creutzfeldt–Jakob disease, and transmission of microorganisms or retroviruses [139]. Therefore, the use of both allogenic and xenogenic material to produce cardiovascular replacements necessitates either crosslinking or decellularization of the tissue. Crosslinking the extracellular matrix, however, results in a fixed, non-living and non-degrading matrix, which will not be

able to grow and remodel and is not suitable as a scaffold for tissue engineering. Alternatively, decellularization of the biological tissues decreases the immunological response without limiting the remodeling capacity, which in turn favorably impacts the long-term graft durability [140]. Various decellularization methods for extracellular matrix are developed and documented [141-144], aiming at the removal of all cellular and nuclear matter while reducing any effects on the integrity and structure of the remaining ECM to preserve the biomechanical characteristics of the native graft [145, 146]. Complete cell removal from the tissue is crucial, as residual cells and cell remnants within the matrix might lead to calcification [147]. Nevertheless, the clinical efficacy of commercially available materials has already been largely positive despite small amounts of remaining DNA [148]. Biological scaffold materials, from which most or all visible cellular material is removed, are suggested to be safe for implantation [143]. It should be noticed that the best method for removing all cellular remnants (meanwhile leaving the extracellular matrix intact) might be different from the most favorable treatment with respect to in-vitro or in-vivo repopulation of the matrix. This because a small pore size, low porosity, and higher density of the collagen network restrict the (seeded) cell adhesion, migration and proliferation [149, 150].

1.3 Myocardial tissue engineering

It is common medical knowledge that every injury in the myocardium results in an irreversible functional deficit as the cardiac muscle has no major potential to regenerate itself. This loss of functional muscular tissue reduces the global work and pumping efficiency of the heart [151]. When the pumping efficiency of the heart is impaired and it cannot pump a sufficient amount of blood to meet the metabolic requirements of the body, which eventually leads to a heart failure. The single most common cause of left-sided cardiac failure is ischemic heart disease followed by acute myocardial infarction. Unfortunately, current interventional or drug therapies cannot adequately control disease progression and eventually heart transplantation is the only treatment option for end-stage heart failure [152]. Due to the widespread lack of organ donors and complications associated with immunosuppressive treatments new strategies to repair the injured cardiac muscle are being investigated leading to the development of several approaches for myocardial tissue engineering in the last decade. In general the field of “cardiac tissue engineering” or “myocardial tissue engineering” aims at restoring the structural and functional damage of the myocardium by use of different techniques and approaches. Overall, “cardiac tissue engineering” comprises the following individual techniques and/or combinations thereof: i) transplantation of cells and/or stem cells aiming at cellular integration or cell-mediated tissue repair, ii) transplantation of in vitro engineered contractile myocardial tissue constructs aiming at their full functional integration, and iii) mobilization of endogenous cells and / or stem cells that induce intrinsic repair phenomena [152-154]. In addition, several approaches have focused on injecting mechanical stabilizers without cells, such as hydrogels, in order to stabilize the myocardial scar and to prevent wall aneurysms and to reduce wall motion abnormalities [155]. As part of this chapter we will mainly focus on the second approach (ii) as this is the only one, where biomaterials

combined with cells will be involved. We will briefly review the materials used in the in vitro engineering of the myocardial tissue.

Myocardial tissue represents one of the most complex tissues of the human body and therefore one of the most difficult ones to engineer in vitro. This fact and the ease of harvest have stimulated approaches using biological tissues as scaffolds for myocardial regeneration [152]. Robinson et al., pioneered this field using 4-layer multilaminate urinary bladder-derived extracellular matrix constructs for the in vivo integration as full thickness left ventricular cardiac patches showing in vivo population with contractile cells as well as rapid biodegradation of the matrix. In a direct comparison with synthetic scaffolds, such as expanded polytetrafluoroethlyene (ePTFE), the native tissue-derived matrices showed superior results [156]. One major obstacle of synthetic tissues was the absence of the typical highly ordered anisotropy present in the native cardiac extracellular matrix, which seems essential to proper myocardial function. Recently, Engelmayer et al. successfully recapitulated this cardiac anisotropy in vitro for synthetic scaffolds using microfabrication techniques to create an accordion-like honeycomb microstructure in polyglycerol sebacate matrices. After culture with neonatal rat cardiomyocytes the engineered matrices showed mechanical properties similar to the native myocardium [157]. In contrary to these studies, several groups have focused on engineering myocardial tissues in vitro even without using any extracellular matrix substrate at all. One approach is the use of cell sheet engineering and cell stacking techniques for the development of contractile cell sheets in vitro. Initially, Shimizu et al. generated pulsatile cardiac tissue grafts via layering of neonatal rat cardiomyocyte cell sheets and stacking to form multilayer sheets [158]. Later studies [159-161] reported the successful in vitro manufacture of vascularized multilayered constructs [160] as well as improved ejection fraction and beneficial left ventricular remodeling after injection in vivo [161]. Similar to these studies the creation of micro-tissue building blocks using the hanging-drop technique for the repair of myocardial tissues has been evaluated extensively in recent years [162, 163]. Recently, the approach has been expanded to different human cell and stem cell sources and principal feasibility was demonstrated after in vivo injection into the border zone of the ischemic myocardium in a porcine model [164, 165]. Taken as a whole, the above reported studies create hope for a potential realization of in vitro engineered functional myocardial replacements in the future. However, when considering the previously mentioned significant progress in the field of vascular tissue engineering with first in-human trials and the long phases of preclinical development, it becomes clear that cardiac tissue engineering is in its fledgling stage and many hurdles remain to be surmounted before concrete clinical therapies will evolve.

1.4 Vascular tissue engineering

The structural design of the scaffolds is a key issue in engineering living vascular substitutes. On the one hand it has to endure the cyclic stresses and strains exerted upon implantation and on the other hand it is also the basis for the seeding cells, an anchor to attach different bioactive molecules and signals. The ideal graft should have and maintain the same compliance as a normal artery and should be flexible and maintaining its

contour. Cell infiltration into the scaffold is one of the prerequisites for successful tissue regeneration and is primarily determined by the scaffold microstructure, i.e. pore size and fiber thickness [166]. Additionally, the microstructure of the scaffold can affect cell phenotype and influences the behavior of the infiltrating cells regarding cell adhesion, spreading, and proliferation [167]. It is also used as an anchor to attach different bioactive molecules and signals that improve specific cell function, such as, pro-angiogenic signals [168]. Finally, "off-the-shelf" availability in various sizes and lengths for emergency care plays an important role, and its handling characteristics should include operative suturability and simplicity of surgical handling.

1.4.1 Polymeric starter matrices in vascular tissue engineering

Different materials have been reported for vascular tissue engineering (table 1), either natural origin, including decellularized matrices [133-135] or collagen [115], or synthetic polymers, such as PET [169] or PGA [58]. Natural-based polymers display no toxic degradation or inflammatory reactions and can be produced from biological sources. On the other hand, synthetic polymers present a higher strength and durability compared to natural polymers, but the bulk degradation might result in local inflammation. Thus, choosing a scaffold material is a critical step in successful vascular tissue engineering. The first TEVG, which was created in 1986 by Weinberg and Bell [115]. They generated cultures of bovine endothelial cells, smooth muscle cells (SMCs) and fibroblasts in layers of collagen gel. However, as the burst pressure of these TEVGs was very low, a polyethylene terephthalate (PET) mesh was added to enhance the burst pressure [115]. During the following years several studies have been conducted to improve the strength of collagen-based constructs by incorporating cells, matrix components, and intracellular biomolecules [170-172]. Girton et al. used glycation to stiffen and strengthen collagen gel construct [173]. Furthermore, un-degradable or degradable meshes were used as matrices [174-176], as well as the utilization of dynamic mechanical stimulation [177]. In 1998, Shinoka et al. reported surgical implantation of TEVGs in lambs, in which scaffolds were constructed from autologous myofibroblasts and endothelial cells seeded onto PGA grafts [57]. This study demonstrated the first vascular graft using autologous cells that yielded a viable structure [57]. In the same year, L'Hereux and coworkers published the generation of a functional blood vessel without a scaffold [178]. Tissue sheets were generated by culturing human SMC and skin fibroblasts to overconfluency and then were fused around a mandrel to generate mechanically robust vessels [178]. This tubular structure mimicked a native artery and displayed a burst pressure of 2594mmHg. However, blood infiltration between vessel layers was observed. One year later Niklason et al. reported the use of PGA scaffold tube seeded with SMC and ECs which was cultured in a bioreactor under flow conditions [58]. Histologic examination of the TEVG presented the vessel wall homogeneously colonized by SMCs. Furthermore, engineered vessels displayed measurable contractions in response to serotonin and prostaglandin. The grafts were patent in vivo up to 4 weeks of observation [58]. Another interesting study based on synthetic biomaterials was published by Dahl et al. They used a PGA scaffold which was seeded by SMCs and subsequently cultured in a bioreactor that delivers cyclic radial strain [179]. After culture period vascular grafts were decellularized to remove antigenic cellular

material and then implanted into baboons as arteriovenous (AV) conduits in nonhuman primates [179]. This study leads to the first U.S. implant of engineered human artery, decellularized allogenic grafts were implanted as AV conduits in few patients with kidney failure [180], which is still ongoing. However, Shinòka and colleagues reported already in 2001 the first application of a TEVG in human [181]. They seeded cells from patient`s peripheral vein on a tubular scaffold made from polycaprolactone-polylactic acid copolymer that was reinforced with PGA. The TEVG was subsequently implanted as a pulmonary artery graft into the patient and seven months after implantation, TEVGs were still functional, without complications or aneurysm [181]. Based on success of this study further tissue engineered constructs were implanted, either as extracardiac total cavopulmonary connections or as tissue engineered patches [182, 183]. There were no complications such as thrombosis or stenosis and all TEVG presented an adequate flow.

Campbell and coworkers reported to grow vessels similar to natural arteries using the in-vivo tissue engineering approach, namely by implanting a silastic tubing into the peritoneal and pleural cavities of animals [19, 20]. Although the produced tissue capsule contained myofibroblasts and was covered by a confluent layer of mesothelial cells, these tubes were rich only in collagen. Collagen provides tensile strength to arteries however, in order to maintain continual pulsatile flow and avoid dilatation, elastin is an essential component [184]. Furthermore, these fibrocollagenous tubes which were formed in-vivo are relatively avascular and thus may decrease exchange between cells and blood after intravascular implantation.

1.4.2 Cell sources for vascular tissue engineering

The basic strategy for vascular tissue engineering includes the design and the production of convenient scaffolds and an appropriate cell choice for cell adhesion, proliferation, and differentiation. Obviously, the first investigated cell sources for vascular tissue engineering were the non-immunogenic autologous endothelial cells (ECs) and smooth muscle cells (SMCs), see also Table 1. ECs are indispensable to maintain vessel wall permeability barrier and to regulate coagulation. ECs serve as mediators of inflammation and additional other physiological processes [185]. Importantly, ECs are the key element to prevent thrombosis in small-diameter vascular grafts and prevent the development of pseudointimal hyperplasia by (i) releasing nitric oxide (NO) and prostacyclin (PGI2) and (ii) producing tissue-type plasminogen activator (t-PA) [101]. Weinberg and Bell published the construction of a TEVG with cultured bovine aortic ECs, SMCs and adventitial fibroblast [115]. Despite these blood vessels structurally mimicked a native artery, they lacked the functions of native arteries due to the burst pressure was very low. Niklason et al. also developed a TEVG based on bovine SMC and ECs seeded onto PGA [58]. Histological analysis displayed that the constructed wall was homogeneously colonized by SMCs. Whatever, McKee et al. supposed that the limiting factor of in vitro TEVG is the restricted proliferative capacity of adult SMCs [4]. They infected human SMCs with human telomerase reverse transcriptase subunit (hTERT) to avoid SMCs ageing [4]. However, safety is still a problem due to the genetic manipulation. Over time alternative cell sources for vascular tissue engineering have become more important. For example, Levenberg and coworkers described the differentiation steps of human ESCs into

ECs forming tube-like structures on matrigel and forming microvessels containing mouse blood when transplanted into servere combined immune deficiency (SCID) mice [30]. Cho et al. used bone marrow derived ECs and SMCs from canine to engineer small-diameter vascular grafts in vitro [186]. Interestingly, Shinòkas` and Matsumuras` groups have also used bone marrow derived cells to repair vessel defects in canine as well as in patients [187-189]. Another study demonstrated the construction of an elastic vessel wall in small diameter using SMCs differentiated from human ASCs [190]. Therefore, the SMCs were seeded in PGA unwoven mesh and following subjected to pulsatile stimulation in a bioreactor for 8 weeks. The engineered vessel wall presented a dense and well-organized structure similar to that of native vessels [190].

1.5 Heart valve tissue engineering

Tissue-engineered heart valves (TEHVs) are proposed as alternative valve replacements to overcome the limitations of current substitutes. Of the three aforementioned tissue-engineering approaches, the in-vitro and the in-situ approach are extensively investigated for development of heart valve replacements using various scaffold materials and cell sources (as listed in Table 2). The third in-vivo tissue-engineering approach, that relies on autologous tissue formation, has also been applied for heart valve engineering by intraperitoneal implantation of either a fixated bovine pericardial pulmonary valve scaffold [16] or mold in the shape of a tri-leaflet valve [17, 18]. However, this approach faces an unbalanced volume fraction of cellular phenotypes, resulting in inadequate remodeling of the valves [191].

1.5.1 Polymeric starter matrices in heart valve tissue engineering

Different types of scaffold material, as introduced in the sections before, are used for heart valve tissue engineering. The scaffold for valve replacement that comes closest to the geometry and structure of the native valve with native-like mechanical and physiological hemodynamics, is the xenograft or allograft (homograft) valve. Obviously, the allograft valve is a more natural option compared to the xenograft valve, as the ideal valve replacement should show similar biomechanical characteristics to the native heart valve. Natural leaflet motion and flow patterns are supposed to avoid stress related calcification and early valve failure [140]. The microstructure of the allograft valve is favorable with respect to proliferation, differentiation, and survival of reseeded cells [144]. The availability of the allograft valve, however, is limited by donor shortage. Due to its anatomic similarity to human valves, the porcine heart valve represents an attractive alternative to allograft valves. Additionally, also xenogenic pericard is investigated as material for valve replacements [192]. However, xenografts are associated with the risk of immunogenic reactions or disease transmission and the availability of homografts is limited [193]. To eliminate immune reactions with xenogenic tissues, they are decellularized before implantation [194] and are either reseeded prior to implantation [195, 196] or expected to become repopulated in-vivo. Although the latter was demonstrated

in animal models [21, 197-199], clinical studies led to contrary reports about cellular infiltration in humans [200-204].

To overcome the limitations of xeno- and homografts, the applicability of various bioresorbable polymers, either synthetic (such as PGA, P4HB and PCL) and natural materials (such as fibrin and collagen) was investigated (Table 2). Seeded with (autologous) cells and subsequently cultured in-vitro, these materials have shown to be feasible for heart valve tissue engineering, with demonstrated functionality in-vitro and in-vivo [164, 165, 205-214]. For example autologous myofibroblast and ECs (or neonatal human dermal fibroblast) have been used to create fibrin-based TEHVs and have been evaluated in sheep up to 3 months. Although the leaflets remodeled in-vivo, the retraction of the leaflets resulted in valvular insufficiency therewith hampering the valve functionality [211, 212, 215, 216]. Also the synthetic bioresorbable polymer PGA showed promising results when combined with P4HB or PLLA [208, 213, 217-219]. When seeded with autologous myofibroblasts, mesenchymal stem cells (MSCs), and ECs valves can be engineered in-vitro that undergo structural and functional remodeling in-vivo without stenosis. However, also in these valves functionality was hampered by trival to moderate regurgitation after 6 weeks due to thickening and retraction [213][214].

1.5.2 Cell sources for heart valve tissue engineering

In the native heart valve, valvular interstitial cells ensure the maintenance of the living tissue with the capability to grow and repair. The two most important functions of these cells are to replicate and to synthesize and remodel the extracellular matrix [220]. Therefore, to prevent in-vivo deterioration, a living valve replacement with regeneration capacity is pursued. It is, however, unclear whether there is a need for a living tissue at time of implantation, or whether circulating cells in the blood or cells from adjacent tissues will be able to repopulate a scaffold in-vivo [203, 204]. Although accumulating evidence suggests that circulating endogenous cells can be recruited in-vivo in animal models [21, 197-199], clinical studies led to contrary reports about cellular infiltration in humans. Although a single case of complete repopulation of the vessel wall and partial repopulation of the distal part of the leaflet was demonstrated recently [200], others observed only sparsely cellular infiltration in the wall [201, 202] and leaflets [203] of decellularized valve tissues implanted in humans. Acellular xenograft valves implanted in children resulted in dramatic structural failure, due to a strong inflammatory response without signs of cell repopulation or endothelialization of either the valve leaflets or the conduit wall [204]. Thus, for clinical application it might be required to preseed the scaffold prior to implantation, or to modulate the scaffold in order to attract the proper endogenous cells in-vivo. Moreover, preseeding may also be necessary to reduce thrombogenicity and reduce the inflammatory reaction.

While it is still unclear whether there is a need for a living implant at the time of implantation, a variety of cell types have been described for in-vitro (re)seeding of synthetic or biological scaffolds, as summarized in Table 2. Experiments comparing allogenic and autologous cells demonstrated that the seeded cells should

be of autologous source to minimize the immune response [163]. However, it was recently shown that also autologous cells can provoke an immune response caused by pro-inflammatory cytokines originating from cell death, or DAMP molecules released by dying cells within the autologous tissue [193, 221].

Vascular derived myofibroblasts and endothelial cells can be harvested from the recipient saphenous vein [222]. Alternatively, cells derived from bone marrow or umbilical cords (blood) and the circulating endothelial progenitor cells show the potential to provide both interstitial and endothelial function and are successfully used to generate heart valves in-vitro Hoerstrup [208, 217-219]. In contrast to vascular cells, these cells can be obtained without surgical intervention, thereby enabling potential adaption into in a routine clinical scenario.

It is clear that the most appropriate cell type for in-vitro preseeding is not established yet. Bone marrow derived MSCs are attractive candidates. These cells are successfully used to reseed decellularized matrices [144, 223] or synthetic scaffolds [208, 209, 224]. MSCs show a remarkable similarity in phenotype compared to valvular interstitial cells [225] and demonstrate anti-thrombogenic [226] and immunosuppressive properties [227]. These cells are favorable for utilization in routine clinical practice, due to their ease of accessibility and handling and their use in allogenic application [228]. Additionally, both the anti-thrombogenic properties of the MSCs [226] and the ability to stimulate in-vivo endothelialization [229], overcome the necessity of preseeding with endothelial cells. Moreover, MSCs are able to differentiate into endothelial cells, fibroblasts or myofibroblasts, and smooth muscle cells [144]. Alternatively, scaffolds (re)seeded with MSCs just prior to implantation, have been successfully implanted in sheep and baboons [223, 230]. Instead of proliferation, differentiation, and extracellular matrix production in-vivo, MSCs may induce the homing and differentiation of autologous host cells, through a paracrine secretion of growth and chemotactic factors enabling tissue regeneration [231].

1.6 The challenges in bench to bedside research

1.6.1 Challenges and limitations in vascular tissue engineering

Despite the variety of accepted biomaterials for clinical applications and successful in-vitro studies for TEVGs, just few reports describe the effective transplantation of tissue-engineered vascular grafts into human patients. In 2001, Shinòka`s group performed the first clinical study using vascular cell-seeded bioresorbable polymer scaffolds (PGA or PLLA 50:50 copolymer ε-caprolactone) in the high-flow low-pressure pulmonary venous system of pediatric patients [181]. Seven months after implantation, TEVGs were still functional, without complications or formation of aneurysms [181]. Nevertheless, few patients showed TEVG stenosis, which was successfully treated with angioplasty [232]. L`Heureux`s group generated a complete autologous in-vitro grown TEVG without a scaffold and implanted these grafts in 10 hemodialysis patients with end-stage renal disease and hemodialysis failure [233]. However, in three cases the TEVGs failed due to thrombosis and/or formation of an aneurysm [233]. Both clinical studies demonstrated encouraging results; however, in the both studies the technologies and autologous cell systems involved are associated with significant

amounts of logistical and regulatory complexity. Therefore, an off-the-shelf alternative would be desirable that could be produced and stored in large numbers and is available for a large number of patients. A potential solution is offered by clinical trials which have been recently started in Poland and the USA, based on PGA scaffolds seeded with SMCs [179, 180]. After culture in a bioreactor, decellularized allogenic grafts were implanted as AV conduits in patients with kidney failure [180] and these trials a still going on. These decellularized grafts can be stored for longer periods and did not cause significant immune response in primates [179]. Also other groups have focused on this approach using different scaffolds and decellularization techniques. In these studies the fast in vivo cellular repopulation evident in the generated matrices was highly interesting [33, 70, 234]. Also for this approach preclinical trials have been performed [33, 70, 234]. However, more sophisticated studies are warranted and an optimal standardized protocol has to be defined by future studies on the way to the clinical realization of the technology.

1.6.2 Challenges and limitations in heart valve tissue engineering

Translation of the completely autologous living TEHVs into clinics faces several challenges such as the ethical and logistic hurdles to overcome with the isolation, culture and use of autologous cells. Therefore, the feasibility and functionality of these autologous living valves, created according to the classical tissue-engineering paradigm, have only been demonstrated in animal studies. One of the limitations for this approach is cell-mediated contraction of the valve leaflets caused by traction forces exerted by the cells. On the one hand, this cell-mediated contraction is functional in ensuring the development of a desirable, highly aligned extracellular matrix [211, 215, 235-237]. However, on the other hand sustained traction forces exerted by the cells may damage the tissue and restrict culture time, but shorter culture times limit collagen and elastin deposition [211]. Furthermore, in valvular tissue-engineering, sustained traction forces can lead to undesirable retraction of the leaflets, which results in valvular regurgitation [163, 164, 212-215]. It has been suggested to avoid the valvular insufficiency by creating longer leaflets, therewith enlarging the coaptation area [215, 238], engineering bi-leaflet valves instead of tri-leaflet valves [211, 238], or applying a bioresorbable support material within the fibrin scaffold [212]. As the observed retraction is mainly cell-mediated, others applied the myosin inhibitor blebbistatin to the TEHV prior to implantation [215]. Although this transiently blocked further cell-induced tissue contraction initially, leaflet retraction reoccurred beyond 4 weeks follow-up in the ovine model. It can be concluded that a proper solution to control these retraction forces is lacking and it remains a challenge to overcome retraction of the tissue engineered leaflets. The cellular stress generation in tissue engineered constructs demonstrated to comprise both a passive and active contribution of the cells [239]. Therefore, retraction of the tissue-engineered leaflets may be prevented by removal of the cellular components. Besides overcoming the cell-mediated retraction of the leaflets, decellularization of the TEHVs enables off-the-shelf availability of the valve replacement [33] demonstrating promising results both in sheep and non-human primate models [70, 240]. Moreover, in these studies also another challenge in the field of heart valve tissue-engineering was tackled, namely the

combination with novel minimally invasive implantation procedures. Although new catheter based implantation techniques are rapidly evolving, the current valve substitutes applicable for these techniques are bioprosthetic in origin, consequently lack repair-, remodeling- and growth capacity, and therewith are restricted to the use in elderly patients. Recently, it was demonstrated that the vulnerable living engineered tissues withstand the necessary crimping of the stented valve replacement to make them suitable for minimally invasive implantation techniques [214, 241, 242], therewith opening the way towards the translation of these minimally invasive replacement techniques also for children and young adults.

The first clinical implementation of TEHVs was performed with decellularized pulmonary allograft valves, reseeded with autologous endothelial progenitor cells, showing improved freedom from re-intervention in contrast to conventional homografts and xenografts. They demonstrated low gradients in follow-up and possibly exhibited adaptive growth (interpreted from the increasing diameter of the tissue-engineered pulmonary valve annulus and reduction of valve regurgitation) [195]. Decellularized allograft valves, reseeded with autologous vascular endothelial cells, demonstrated uncompromised follow-up of 10 years, with excellent hemodynamic performance [196]. However, the need for valve replacements exceeds the supply of donor valves. Therefore, also the largely available decellularized xenogenic pulmonary valves, reseeded with autologous vascular endothelial cells, have been introduced for right ventricular outflow tract reconstruction, with excellent early and midterm results [243]. Moreover, the short- and mid-term performance of non-seeded decellularized xenograft valves in children and patients with congenital heart disease were recently reported to meet the performance of other currently available implants [200]. Nevertheless, as mentioned earlier, the first clinical non-seeded applications in children resulted in dramatic structural failure of the heart valves, due to strong inflammatory responses [204]. Additionally, complete destruction of a decellularized pulmonary xenograft valve was reported in a single case [244].

1.7 Conclusions

For the in-vitro manufacture of cardiovascular bioengineered constructs several different materials have been used over the last decades. Besides the use of surface treated or modified native tissues, also natural and fully synthetic polymer scaffolds have been used to serve as a basis for tissue engineering processes. As the scaffold plays a crucial role in successful designing of tissue engineering constructs, the choice of material directly influences the outcome. Natural-based polymers display no toxic degradation or inflammatory reactions and can be produced from biological sources. On the other hand, the main advantage of synthetic polymers is certainly the chance to actively control the polymer composition and therefore to modify the durability, strength, and biodegradation behavior of the deriving constructs. The main advantage of complete natural matrices represents the unique functional characteristics currently only present in native tissues. Composite biomaterials have the potential to overcome the current predicament of having to choose between either synthetics or natural tissues. Therefore, despite a variety of materials have been validated, future effort should focus on perfecting composite materials to take full advantage of the best properties.

So far, scientists in the field of heart valve tissue engineering have not been able to create synthetic matrices with the same unique functional characteristics of a native heart valve. The latest bioengineered heart valve constructs still lack the heterogenous anisotropic microstructural organization of the native tissue, and thereby inferior to the very distinct physiological function of the native valve with contractile elements in systole and durable load bearing behavior in diastole. Moreover, they do not contain all components of the native leaflets and lack the microstructural elements presents in the native-analogous organization. This amplifies that as long as natural tissues are superior to synthetic scaffolds in terms of functional behavior, the use and clinical translation of synthetic polymer matrix-based tissue engineered cardiovascular constructs remains to be rather future hope than scientific reality. On the other hand, given the widespread scarcity of donor organs and the immunological, infectious as well as ethical hurdles of cross-species transplants, only synthetic or human derived natural off-the-shelf products hold the potential to offer therapeutic solutions for the tremendously increasing numbers of cardiovascular patients worldwide. This implies that scientists will and have to stay committed to designing improved polymeric starter matrices for the replacement of cardiovascular tissues. However, it seems essential that future synthetic scaffolds not only copy native tissues in their composition of structural components, but also duplicate their microstructural organization. In the coming decades research will most likely focus on intelligent, off-the-shelf scaffolds that make use of the regenerative capacity of the human body by attracting endogenous cells. Obviously, long-term (pre-clinical) studies are compulsory to evaluate the remodeling of these optimized materials towards native like tissues. Nevertheless, bioresorbable scaffolds have large potential to eventually replace the use of the current synthetic and fixed-biological grafts and function as therapeutic replacement structures in the future.

1.8 Aim of the study

Tissue engineering (TE) is a discipline with the intention to repair, replace or regenerate injured tissues and organs. The three essential elements of in vitro TE comprise i) the cells that are seeded onto the graft, ii) the scaffold onto which the extracellular matrix forms, and iii) the biological and mechanical stimuli that promote new tissue formation and maintenance**.** Over the last decades TE has made great strides forward and different scaffold materials and cell sources have been validated with a main focus on their therapeutic potential for regenerative medicine. In combination with TE, stem cells are associated with a number of promises in further advancing contemporary medicine. An ideal cell source for human therapeutic and disease modeling applications should be non-immunogenic, functional, and easy to isolate and expand. Therefore, induced pluripotent stem cells from human peripheral blood are a promising source for establishing robust, patient-specific tissue models for studying the pathogenesis of vascular disease as well as for developing novel therapeutic applications based on *in-vitro* TE.

While the engineered living substitute develops, the biocompatible scaffold should degrade without leaving remnants in the body, requiring a so-called biodegradable starter matrix (scaffold). Synthetic polymers have been extensively used for TE, given their high durability, flexibility, and mechanical strength. Additionally, production conditions of synthetic polymers can be tightly controlled, hence making mechanical and physical properties of the material predictable, reproducible and precisely defined . These characteristics make synthetic polymers to an interesting raw material for scaffold fabrication.

Therefore, the aim of the studies contained in this thesis is to (a) compare three commonly used synthetic polymers (P4HB, PLA, and PCL) for *in-vitro* TE applications under static and dynamic conditions, (b) reprogram and characterize peripheral blood mononuclear cells (PBMCs) into hiPSCs, (b) differentiated and characterize hiPSCs into smooth muscle cells and endothelial cells for *in-vitro* tissue engineering, (c) assess and compare the tissue-enginneered vascular grafts cultured under static and dynamic conditions.

Acknowledgements

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013] under grant agreement n^{o}. 242008 and grant agreement n^{o}. 626976.

Table 1: Tissue-engineering of vascular grafts: *scaffold materials, cell sources, (pre-) clinical studies, and main conclusions.*

Scaffold	Cell source (& in-vitro culture period after seeding, if applicable)	(Pre-) clinical study (follow-up period)	Conclusions and references
Synthetic materials			
PGA	Bovine SMC and EC	Miniature swine (up to 24 days)	In-vivo, lack of mature elastin and polymer remnants [245]
	Autologous myoFBs and EC	In lambs (up to 24 weeks)	In-vivo, growth and development of endothelial lining and ECM [246]
	Human SMC (decellularization after culture)	In nonhuman primates (up to 6 months)	In-vivo, arteriovenous conduits, can be stored at least 12 months, no aneurysmal dilatation, no calcification, no substantial intinam hyperplasia, clinical trial followed [247]
	Canine SMC (decellularization after culture)	In canine (up to 12 months)	In-vivo, excellent long-term patency, no stenosis or dilatation, no intimal hyperplasia [247]
	Human SMCs; Pulsatile stretching, decellularization	In human	AV shunts for dialysis (ClinicalTrials.gov Identifier: NCT01744418 and NCT01840956)
PGA+PHA	bovine carotid artery cells	In lambs (up to 5 months)	In-vivo, remained patent, no aneurysms [142]
PGA+P4HB	Lamb vascular cells	In lambs (up to 100 weeks)	In-vivo, growth and remodeling capacity of implants, no thrombus, calcification, stenosis, or aneurysm [248]
PCL-PLA polymer with PGA	Autologous BM-MNC, no culture	In human	Extracardiac cavopulmonary conduits [249-251]
PCL	n.a.	In rats (up to 18 months)	In-vivo, no aneurysm, no calcification, polymers remnants [211]
	n.a.	In rats (up to 18 months)	In-vivo, until 6 months: perfect patency, after 6 months: regression [252]
Natural materials			
Fibrin	Lamb SMC		In-vitro, two-layer grafts, SMCs only in inner layer [15]

	Human dermal FB		In-vitro, entrapment of FB in fibrin gel, burst pressures (1400-1600 mmHg) and compliance comparable to native arteries [253]
Collagen	Bovine SMC, FBs and EC		In-vitro, mimicked a native artery, burst pressure very low [254]
Chitosan	Rabbit vascular SMC		In-vitro, proper swelling property, burst strength: 4000mmHG, high suture-retention strength [255]
Collagen/ elastin tubular scaffolds	Human vascular SMC		In-vitro, circumferential orientation of SMCs was uniformly distributed [256]
Biological decellularized materials			
Human umbilical artery		In rat (up to 8 weeks)	In-vivo, 5 rats died within few hours after implantation (thrombosis), 6 TEVGs remained patent [257]

Table 2: Tissue-engineering of heart valves: *scaffold materials, cell sources, (pre-) clinical studies, and main conclusions.*

Scaffold	Cell source (& in-vitro culture period after seeding, if applicable)	(Pre-) clinical study (follow-up period)	Conclusions and references
Synthetic materials			
PHA	Autologous myoFB and EC	In sheep (up to 17 weeks)	In-vivo functionality with minimal regurgitation; laminated fibrous tissue with predominant glycosaminoclycans [207].
PLA & PLGA	Human dermal FB and bovine aortic EC or ovine myoFB and EC (2 weeks)	n.a.	In-vitro p.o.p. of human TEHV [247].
P4HB	Autologous vascular medial and EC (8 days)	In sheep (up to 24 weeks)	In-vivo functionality of viable leaflets without thrombus; with mild, non-progressive valvular regurgitation [258].
	Human UC-blood-DPC (3 weeks)	n.a.	In-vitro generation of viable TEHV from CD133+ cells [219].
PGA	Ovine (autologous) myoFB and EC (>2 weeks)	In sheep (up to 11 weeks)	In-vivo p.o.p. of TEHV based on synthetic scaffolds; no stenosis; trivial (autografts) and moderate (allogenic valves) regurgitation; suggest superiority of autograft tissue [163, 164].
PGA & P4HB	Human myoFB and EC (4 weeks)	n.a.	In-vitro p.o.p. of TEHV for systemic application [71].
	Human marrow stromal cells (3 weeks)	n.a.	In-vitro p.o.p. of TEHV based on human MSC [208].
	Human UC-DPC (3 weeks)	n.a.	In-vitro p.o.p. of TEHV based on human umbilical cord-derived progenitor cells [259].
	Human amniotic fluid derived progenitor cells (3 weeks)	n.a.	In-vitro p.o.p. of TEHV based on human amniotic fluid derived progenitor cells [217].
	Ovine MSC (3 weeks)	n.a.	In-vitro p.o.p. of TEHV based on ovine MSC [224]
	Ovine peripheral blood EPC (3 weeks)	n.a.	In-vitro p.o.p. ovine peripheral blood EPC as single cell source for TEHV [218].
	Autologous myoFB and EC (3 weeks)	In sheep (up to 20 weeks	In-vivo functionality; mobile leaflets no stenosis, thrombus, or aneurysm; remodeling towards native valves in microstructure,

			mechanical properties, and extracellular matrix [205].
	Autologous myoFB and EC (up to 4 weeks)	In sheep (up to 8 weeks)	In-vivo p.o.p. of trans-apical implantation of TEHV; in-vivo pulmonary functionality with mobile, but thickened leaflets [214].
	Autologous MSC, cultured 4 weeks)	In sheep (up to 8 months)	In-vivo p.o.p. of TEHV based on MSC, with trival to mild regurgitation at implantation and remodeling towards native valves [209].
	Autologous bone marrow stromal cells	In non-human primates (up to 4 weeks)	In-vivo p.o.p. of minimally invasive one-step approach (isolation, seeding, and subsequent implantation); remodeling to endothelialized anisotropic tissues [230].
	Autologous bone marrow stromal cells	In sheep (acute study)	In-vivo p.o.p. of trans-apical implantation of TEHV in the systemic circulation [260].
PGA & PLLA	Autologous MSC (1 month)	In sheep (up to 20 weeks)	In-vivo functionality with trival to moderate regurgitation after 6 weeks due to thickening and retraction; valves undergo structural and functional remodeling without stenosis [213].
PCL	n.a.	n.a.	In-vitro p.o.p of functionality of electrospun scaffolds [261, 262].
	Human myoFB (3 weeks)	n.a.	In-vitro testing of knitted scaffold for TEHV shows low seeding efficiency, but better strength compared to electro -spun scaffold [263].
Natural materials			
Fibrin	Neonatal human dermal FB (3-5 weeks)	In sheep (up to 8 weeks)	In-vitro p.o.p. for aortic pressures, and in-vivo p.o.p. of fibrin-based TEHV with increasing regurgitation (trival to moderate) at 4 weeks; at 8 weeks complete retraction hampered functionality [211, 215, 216].
	Autologous myofibroblast and EC (4 weeks)	In sheep (up to 3 months)	In-vivo p.o.p. of fibrin-based TEHV with remodeling leaflets, but retraction resulted in valvular insufficiency [212].
Fibrin & poly-l-lysine	Human myoFB	n.a.	In-vitro p.o.p. of fibrin-based TEHV, external fixation is required to control leaflet retraction [264].
Collagen	FB (up to 8 weeks)	n.a.	In-vitro p.o.p. of TEHV based on directed collagen gel shrinkage [252].
Biological decellularized materials			
Human pulmonary valve	n.a.	In human	SynerGraft treatment reduced tissue antigen expression but did not alter biomechanics or strength; lack of panel reactive antibody response [137].

	n.a.	In human (up to 5 years)	Moderate regurgitation; showed improved freedom from explantation, and low gradients compared to xeno- and homografts [195].
	Autologous vascular EC	In human (up to 10 years)	Excellent hemodynamic performance during mid-term follow-up. Biopsy of wall reveled recellularization [196, 202, 243, 255].
	Autologous EPC (3 weeks)	In 2 children (up to 3,5 years)	Good functionality with decreased regurgitation (mild to trival) and increased pulmonary annulus diameter [265].
Human aortic valve		In human	Low transvalvular gradients similar to conventional homografts, but with low panel reactive antibody response [266].
Ovine pulmonary valve		In sheep (up to 11 months)	In-vivo proof of completely endothelialization (after 6 months) and up to 80% leaflet recellularization with interstitial cells after 11 months (with expression of α-SMA similar to native valve leaflets); no calcification; no regurgitation [137, 197, 198, 267, 268].
		In sheep (up to 1 year)	In-vivo recellularization of basal region of cusp with myoFB; no calcification; trivial regurgitation [138].
	With or without autologous EC (5 days)	In sheep (up to 6 months)	In-vivo endothelialization and partially cellular infiltration of leaflets; no regurgitation or calcification; no difference between seeded and non-seeded groups [267-269].
	Autologous myoFB and EC (8 days)	In sheep (up to 3 months)	In-vivo functionality; complete endothelialization; no calcification of valve [206].
Ovine aortic valve		In sheep (up to 9 months	In-vivo incomplete endothelialization of leaflet; cellular infiltration in wall and very sparse in leaflet; no calcification [270].
Porcine pericardium	n.a.	In rat (up to 6 weeks)	In-vivo PGG-treated pericardium did not calcify; infiltration by host FB with subsequent matrix remodeling [113].
	Human MSC (8 days)	n.a.	In-vitro p.o.p. of layered fibrous and spongiosa scaffolds (pericardium and pulmonary artery) [192].
Porcine pulmonary valve	n.a.	In human children	Structural valve failure between 7 days and 1 year due to strong inflammatory responses; no repopulation [204].
	n.a.	In human (up to 5 years)	Valve performance superior to currently available implants; no calcification [200].
	Autologous EC	In human (up to 5 years)	Excellent hemodynamic performance; no calcification; no difference compared to allogenic decellularized valves [243].
	Autologous myofibroblast and EC (16 days)	In sheep (up to 4 weeks)	In-vitro and in-vivo p.o.p. of TEHV for percutanous implantation; in-vivo functionality; expression of α-SMA in leaflets [241, 271, 272].

	Autologous bone marrow mononuclear cells or MSC, injected prior to implantation	In sheep (up to 4 months)	In-vivo endothelialization and cellular infiltration in both groups; valve calcification, thickening, retraction and inflammation in BMMC group; thin leaflets, no calcification, lower transvalvular gradients and remodeling towards native valves in MSC group [223].
Porcine aortic valve	n.a.	Subdermal in rat (up to 6 weeks)	In-vivo calcification was reduced by TRI-COL / nuclease treatment [141].
	n.a.	In sheep (up to 5 months)	In-vivo proof of functionality untill explantation; ingrowth of host fibroblastoid cells; no calcification [197].
	Human vascular EC (3 days)	n.a.	In-vitro p.o.p. of TEHV by seeding decellularized matrices [273].
	Human MSC (4 weeks)	n.a.	In-vitro proof of human MPC to infiltrate acellular porcine valve matrix under static conditions; reseeded cells also expressed osteogenic markers [274].
	Human neonatal dermal FB (8 weeks)	n.a.	In-vitro proof of gradual population of accelular matrix with viable cells that produce matrix [275].

Abbreviations used in table 1&2

Scaffold	*Cell source*	*Others*
P4HB = poly-4-hydroxybutyra	FB = fibroblasts	n.a. = not applicable
PHA = polyhydroxy-alcanoate	EC = endothelial cells	p.o.p. = proof of principle
PGA = polyglycolic acid	MSC = mesenchymal stem cells	
PLA = polylactic acid	EPC = endothelial progenitor cells	
PLGA = copolymer of	UC-DPC = umbilical cord –	
PGA and PLA	derived progenitor cells	
PCL = polycaprolactone		

1.9 References

1. World Health Organization (WHO) Fact sheet N°317. 2013 Cardiovascular diseases (CVDs). http://www.who.int/mediacentre/factsheets/fs317/en/ (accessed March 2014).
2. Rosengart, T.K., T. Feldman, M.A. Borger, T.A.J. Vassiliades, A.M. Gillinov, K.J. Hoercher, A. Vahanian, R.O. Bonow, W. O'Neill, A.H.A.C.o.C.S.a. Anesthesia, A.H.A.C.o.C. Cardiology, F.G.a.T.B.I.W. Group, and Q.o.C.a.O.R.I.W. Group, Percutaneous and minimally invasive valve procedures: a scientific statement from the American Heart Association Council on Cardiovascular Surgery and Anesthesia, Council on Clinical Cardiology, Functional Genomics and Translational Biology Interdisciplinary Working Group, and Quality of Care and Outcomes Research Interdisciplinary Working Group. Circulation, 2008. 117(13): p. 1750-1767.
3. Dave, T., J. Ezhilan, H. Vasnawala, and V. Somani, Plaque regression and plaque stabilisation in cardiovascular diseases. Indian J Endocrinol Metab, 2013. 17(6): p. 983-989.
4. McKee, J.A., S.S. Banik, M.J. Boyer, N.M. Hamad, J.H. Lawson, L.E. Niklason, and C.M. Counter, Human arteries engineered in vitro. EMBO Rep, 2003. 4(6): p. 633-8.
5. Verma, S., P.E. Szmitko, R.D. Weisel, D. Bonneau, D. Latter, L. Errett, Y. LeClerc, and S.E. Fremes, Should radial arteries be used routinely for coronary artery bypass grafting? Circulation, 2004. 110(5): p. e40-6.
6. Rathore, A., M. Cleary, Y. Naito, K. Rocco, and C. Breuer, Development of tissue engineered vascular grafts and application of nanomedicine. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2012. 4(3): p. 257-72.
7. Peck, M., D. Gebhart, N. Dusserre, T.N. McAllister, and N. L'Heureux, The evolution of vascular tissue engineering and current state of the art. Cells Tissues Organs, 2012. 195(1-2): p. 144-58.
8. Pibarot, P. and J.G. Dumesnil, Prosthetic heart valves: selection of the optimal prosthesis and long-term management. Circulation, 2009. 119(7): p. 1034-1048.
9. Yacoub, M.H. and J.J.M. Takkenberg, Will heart valve tissue engineering change the world? Nat.Clin.Pract.Cardiovasc.Med., 2005. 2(2): p. 60-61.
10. Clarke, D.R., Value, viability, and valves. J.Thorac.Cardiovasc.Surg., 2002. 124(1): p. 1-6.
11. Schoen, F.J. and R.J. Levy, Calcification of tissue heart valve substitutes: progress toward understanding and prevention. Ann.Thorac.Surg., 2005. 79(3): p. 1072-1080.
12. Skalak, R. and C.F. Fox. Tissue Engineering. 1988. Granlibakken, Lake Tahoe: New York: Liss.
13. Langer, R. and J.P. Vacanti, Tissue engineering. Science, 1993. 260(5110): p. 920-926.
14. Mendelson, K. and F.J. Schoen, Heart valve tissue engineering: concepts, approaches, progress, and challenges. Ann.Biomed.Eng, 2006. 34(12): p. 1799-1819.
15. Schoen, F.J., New frontiers in the pathology and therapy of heart valve disease: 2006 Society for Cardiovascular Pathology, Distinguished Achievement Award Lecture, United States-Canadian Academy of Pathology, Atlanta, GA, February 12, 2006. Cardiovasc.Pathol., 2006. 15(5): p. 271-279.
16. Visscher, G.d., I. Vranken, A. Lebacq, K. C.van, J. Ganame, E. Verbeken, and W. Flameng, In vivo cellularization of a cross-linked matrix by intraperitoneal implantation: a new tool in heart valve tissue engineering. Eur.Heart J., 2007. 28(11): p. 1389-1396.
17. Hayashida, K., K. Kanda, H. Yaku, J. Ando, and Y. Nakayama, Development of an in vivo tissue-engineered, autologous heart valve (the biovalve): preparation of a prototype model. J.Thorac.Cardiovasc.Surg., 2007. 134(1): p. 152-159.
18. Yamanami, M., Y. Yahata, M. Uechi, M. Fujiwara, H. Ishibashi-Ueda, K. Kanda, T. Watanabe, T. Tajikawa, K. Ohba, H. Yaku, and Y. Nakayama, Development of a completely autologous valved conduit with the sinus of Valsalva using in-body tissue architecture technology: a pilot study in pulmonary valve replacement in a beagle model. Circulation, 2010. 122(11 Suppl): p. S100-S106.
19. Campbell, J.H., J.L. Efendy, and G.R. Campbell, Novel vascular graft grown within recipient's own peritoneal cavity. Circ Res, 1999. 85(12): p. 1173-8.
20. Chue, W.L., G.R. Campbell, N. Caplice, A. Muhammed, C.L. Berry, A.C. Thomas, M.B. Bennett, and J.H. Campbell, Dog peritoneal and pleural cavities as bioreactors to grow autologous vascular grafts. J Vasc Surg, 2004. 39(4): p. 859-67.

21. Elkins, R.C., P.E. Dawson, S. Goldstein, S.P. Walsh, and K.S. Black, Decellularized human valve allografts. The Annals of Thoracic Surgery, 2001. 71(90050): p. S428-S432.
22. Mol, A., A.I. Smits, C.V. Bouten, and F.P. Baaijens, Tissue engineering of heart valves: advances and current challenges. Expert.Rev.Med.Devices, 2009. 6(3): p. 259-275.
23. Catto V., F.S., Freddi G., and Tanzi M.C., Vascular Tissue Engineering: Recent Advances in Small Diameter Blood Vessel Regeneration. ISRN Vascular Medicine, 2014. 2014.
24. Levenberg, S., Engineering blood vessels from stem cells: recent advances and applications. Current Opinion in Biotechnology, 2005. 16(5): p. 516-523.
25. Cohen, S., L. Leshanski, and J. Itskovitz-Eldor, Tissue engineering using human embryonic stem cells. Stem Cell Tools and Other Experimental Protocols, 2006. 420: p. 303-315.
26. Evans, M.J. and M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos. Nature, 1981. 292(5819): p. 154-6.
27. Martin, G.R., Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A, 1981. 78(12): p. 7634-8.
28. Stock, U.A. and J.P. Vacanti, Cardiovascular physiology during fetal development and implications for tissue engineering. Tissue Eng, 2001. 7(1): p. 1-7.
29. Taylor, P.M., P. Batten, N.J. Brand, P.S. Thomas, and M.H. Yacoub, The cardiac valve interstitial cell. Int.J.Biochem.Cell Biol., 2003. 35(2): p. 113-118.
30. Levenberg, S., J.S. Golub, M. Amit, J. Itskovitz-Eldor, and R. Langer, Endothelial cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A, 2002. 99(7): p. 4391-6.
31. Mimeault, M. and S.K. Batra, Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. Stem Cells, 2006. 24(11): p. 2319-45.
32. Weber, B. and S.P. Hoerstrup, Regenerating Heart Valves. Regenerating the Heart: Stem Cells and the Cardiovascular System, 2011: p. 403-442.
33. Dijkman, P.E., A. Driessen-Mol, L. Frese, S.P. Hoerstrup, and F.P.T. Baaijens, Decellularized homologous tissue-engineered heart valves as off-the-shelf alternatives to xeno- and homografts. Biomaterials, 2012. 33(18): p. 4545-4554.
34. Takahashi, K. and S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 2006. 126(4): p. 663-76.
35. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell, 2007. 131(5): p. 861-72.
36. Hibino, N., D.R. Duncan, A. Nalbandian, T. Yi, Y. Qyang, T. Shinoka, and C.K. Breuer, Evaluation of the use of an induced puripotent stem cell sheet for the construction of tissue-engineered vascular grafts. J Thorac Cardiovasc Surg, 2012. 143(3): p. 696-703.
37. Schoen, F.J., Heart valve tissue engineering: quo vadis? Curr Opin Biotechnol, 2011. 22(5): p. 698-705.
38. Kannan, R.Y., H.J. Salacinski, P.E. Butler, G. Hamilton, and A.M. Seifalian, Current status of prosthetic bypass grafts: a review. J Biomed Mater Res B Appl Biomater, 2005. 74(1): p. 570-81.
39. Wang, S., A.S. Gupta, S. Sagnella, P.M. Barendt, K. Kottke-Marchant, and R.E. Marchant, Biomimetic fluorocarbon surfactant polymers reduce platelet adhesion on PTFE/ePTFE surfaces. J Biomater Sci Polym Ed, 2009. 20(5-6): p. 619-35.
40. Yashiro, B., M. Shoda, Y. Tomizawa, T. Manaka, and N. Hagiwara, Long-term results of a cardiovascular implantable electronic device wrapped with an expanded polytetrafluoroethylene sheet. J Artif Organs, 2012. 15(3): p. 244-9.
41. Johnson, W.C. and K.K. Lee, A comparative evaluation of polytetrafluoroethylene, umbilical vein, and saphenous vein bypass grafts for femoral-popliteal above-knee revascularization: a prospective randomized Department of Veterans Affairs cooperative study. J Vasc Surg, 2000. 32(2): p. 268-77.
42. Xue, L. and H.P. Greisler, Biomaterials in the development and future of vascular grafts. J Vasc Surg, 2003. 37(2): p. 472-80.
43. Bos, G.W., A.A. Poot, T. Beugeling, W.G. van Aken, and J. Feijen, Small-diameter vascular graft prostheses: Current status. Archives of Physiology and Biochemistry, 1998. 106(2): p. 100-115.
44. Zdrahala, R.J., Small caliber vascular grafts .2. Polyurethanes revisited. Journal of Biomaterials Applications, 1996. 11(1): p. 37-61.

45. Nakagawa, Y., K. Ota, Y. Sato, S. Teraoka, and T. Agishi, Clinical trial of new polyurethane vascular grafts for hemodialysis: Compared with expanded polytetrafluoroethylene grafts. Artificial Organs, 1995. 19(12): p. 1227-1232.
46. Middleton, I.P., K.G. Al-Lamee, and J. Paprotny, Professor Bamford's research in the field of biomaterials. Journal of Biomaterials Science-Polymer Edition, 2000. 11(12): p. 1287-1306.
47. James, K. and J. Kohn, New biomaterials for tissue engineering. Mrs Bulletin, 1996. 21(11): p. 22-26.
48. Lyu, S. and D. Untereker, Degradability of polymers for implantable biomedical devices. Int J Mol Sci, 2009. 10(9): p. 4033-65.
49. Vert, M., Bioresorbable Polymers for Temporary Therapeutic Applications. Angewandte Makromolekulare Chemie, 1989. 166: p. 155-168.
50. Vert, M., S.M. Li, G. Spenlehauer, and P. Guerin, Bioresorbability and Biocompatibility of Aliphatic Polyesters. Journal of Materials Science-Materials in Medicine, 1992. 3(6): p. 432-446.
51. Carothers, W.H. and D.D. Coffman, The thermal decomposition of sodium ethyl. Journal of the American Chemical Society, 1929. 51: p. 588-593.
52. Athanasiou, K.A., C.M. Agrawal, F.A. Barber, and S.S. Burkhart, Orthopaedic applications for PLA-PGA biodegradable polymers. Arthroscopy, 1998. 14(7): p. 726-737.
53. Engelberg, I. and J. Kohn, Physicomechanical Properties of Degradable Polymers Used in Medical Applications - a Comparative-Study. Biomaterials, 1991. 12(3): p. 292-304.
54. Couet, F., N. Rajan, and D. Mantovani, Macromolecular biomaterials for scaffold-based vascular tissue engineering. Macromol Biosci, 2007. 7(5): p. 701-18.
55. Pankajakshan, D. and D.K. Agrawal, Scaffolds in tissue engineering of blood vessels. Can J Physiol Pharmacol, 2010. 88(9): p. 855-73.
56. Thomas, L.V., V. Lekshmi, and P.D. Nair, Tissue engineered vascular grafts--preclinical aspects. Int J Cardiol, 2013. 167(4): p. 1091-100.
57. Shinoka, T., D. Shum-Tim, P.X. Ma, R.E. Tanel, N. Isogai, R. Langer, J.P. Vacanti, and J.E. Mayer, Jr., Creation of viable pulmonary artery autografts through tissue engineering. J Thorac Cardiovasc Surg, 1998. 115(3): p. 536-45; discussion 545-6.
58. Niklason, L.E., J. Gao, W.M. Abbott, K.K. Hirschi, S. Houser, R. Marini, and R. Langer, Functional arteries grown in vitro. Science, 1999. 284(5413): p. 489-493.
59. Wu, X., E. Rabkin-Aikawa, K.J. Guleserian, T.E. Perry, Y. Masuda, F.W.H. Sutherland, F.J. Schoen, J.E. Mayer, and J. Bischoff, Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. American Journal of Physiology-Heart and Circulatory Physiology, 2004. 287(2): p. H480-H487.
60. Matsumura, G., N. Nitta, S. Matsuda, Y. Sakamoto, N. Isayama, K. Yamazaki, and Y. Ikada, Long-Term Results of Cell-Free Biodegradable Scaffolds for In Situ Tissue-Engineering Vasculature: In a Canine Inferior Vena Cava Model. Plos One, 2012. 7(4).
61. Watanabe, M., T. Shin'oka, S. Tohyama, N. Hibino, T. Konuma, G. Matsumura, Y. Kosaka, T. Ishida, Y. Imai, M. Yamakawa, Y. Ikada, and S. Morita, Tissue-engineered vascular autograft: inferior vena cava replacement in a dog model. Tissue Eng, 2001. 7(4): p. 429-39.
62. Li, H., R. Du, and J. Chang, Fabrication, characterization, and in vitro degradation of composite scaffolds based on PHBV and bioactive glass. J Biomater Appl, 2005. 20(2): p. 137-55.
63. Chen, G.Q. and Q. Wu, The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials, 2005. 26(33): p. 6565-78.
64. Rezwan, K., Q.Z. Chen, J.J. Blaker, and A.R. Boccaccini, Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials, 2006. 27(18): p. 3413-31.
65. Shum-Tim, D., U. Stock, J. Hrkach, T. Shinoka, J. Lien, M.A. Moses, A. Stamp, G. Taylor, A.M. Moran, W. Landis, R. Langer, J.P. Vacanti, and J.E. Mayer, Jr., Tissue engineering of autologous aorta using a new biodegradable polymer. Ann Thorac Surg, 1999. 68(6): p. 2298-304; discussion 2305.
66. Schmidt, D., U.A. Stock, and S.P. Hoerstrup, Tissue engineering of heart valves using decellularized xenogeneic or polymeric starter matrices. Philos Trans R Soc Lond B Biol Sci, 2007. 362(1484): p. 1505-12.
67. Hoerstrup, S.P., R. Sodian, S. Daebritz, J. Wang, E.A. Bacha, D.P. Martin, A.M. Moran, K.J. Guleserian, J.S. Sperling, S. Kaushal, J.P. Vacanti, F.J. Schoen, and J.E. Mayer, Jr., Functional living trileaflet heart valves grown in vitro. Circulation, 2000. 102(19 Suppl 3): p. III44-9.

68. Rabkin, E., S.P. Hoerstrup, M. Aikawa, J.E. Mayer, Jr., and F.J. Schoen, Evolution of cell phenotype and extracellular matrix in tissue-engineered heart valves during in-vitro maturation and in-vivo remodeling. J Heart Valve Dis, 2002. 11(3): p. 308-14; discussion 314.
69. Stekelenburg, M., M.C. Rutten, L.H. Snoeckx, and F.P. Baaijens, Dynamic straining combined with fibrin gel cell seeding improves strength of tissue-engineered small-diameter vascular grafts. Tissue Eng Part A, 2009. 15(5): p. 1081-9.
70. Weber, B., P.E. Dijkman, J. Scherman, B. Sanders, M.Y. Emmert, J. Grunenfelder, R. Verbeek, M. Bracher, M. Black, T. Franz, J. Kortsmit, P. Modregger, S. Peter, M. Stampanoni, J. Robert, D. Kehl, M. van Doeselaar, M. Schweiger, C.E. Brokopp, T. Walchli, V. Falk, P. Zilla, A. Driessen-Mol, F.P. Baaijens, and S.P. Hoerstrup, Off-the-shelf human decellularized tissue-engineered heart valves in a non-human primate model. Biomaterials, 2013. 34(30): p. 7269-80.
71. Mol, A., M.C. Rutten, N.J. Driessen, C.V. Bouten, G. Zund, F.P. Baaijens, and S.P. Hoerstrup, Autologous human tissue-engineered heart valves: prospects for systemic application. Circulation, 2006. 114(1 Suppl): p. I152-8.
72. Chen, G.P., T. Sato, T. Ushida, R. Hirochika, Y. Shirasaki, N. Ochiai, and T. Tateishi, The use of a novel PLGA fiber/collagen composite web as a scaffold for engineering of articular cartilage tissue with adjustable thickness. Journal of Biomedical Materials Research Part A, 2003. 67A(4): p. 1170-1180.
73. Zhang, Y. and M.Q. Zhang, Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. Journal of Biomedical Materials Research, 2001. 55(3): p. 304-312.
74. Kim, H.W., H.E. Kim, and V. Salih, Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin-hydroxyapatite for tissue engineering scaffolds. Biomaterials, 2005. 26(25): p. 5221-5230.
75. Cascone, M.G., N. Barbani, C. Cristallini, P. Giusti, G. Ciardelli, and L. Lazzeri, Bioartificial polymeric materials based on polysaccharides. Journal of Biomaterials Science-Polymer Edition, 2001. 12(3): p. 267-281.
76. Di Martino, A., M. Sittinger, and M.V. Risbud, Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials, 2005. 26(30): p. 5983-90.
77. Chandy, T. and C.P. Sharma, Chitosan--as a biomaterial. Biomater Artif Cells Artif Organs, 1990. 18(1): p. 1-24.
78. Suh, J.K. and H.W. Matthew, Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials, 2000. 21(24): p. 2589-98.
79. Madihally, S.V. and H.W.T. Matthew, Porous chitosan scaffolds for tissue engineering. Biomaterials, 1999. 20(12): p. 1133-1142.
80. Chuang, W.Y., T.H. Young, C.H. Yao, and W.Y. Chiu, Properties of the poly(vinyl alcohol)/chitosan blend and its effect on the culture of fibroblast in vitro. Biomaterials, 1999. 20(16): p. 1479-87.
81. Jiang, T., S.G. Kumbar, L.S. Nair, and C.T. Laurencin, Biologically active chitosan systems for tissue engineering and regenerative medicine. Curr Top Med Chem, 2008. 8(4): p. 354-64.
82. Berger, J., M. Reist, J.M. Mayer, O. Felt, and R. Gurny, Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm, 2004. 57(1): p. 35-52.
83. Madihally, S.V. and H.W. Matthew, Porous chitosan scaffolds for tissue engineering. Biomaterials, 1999. 20(12): p. 1133-42.
84. Zhang, L., Q. Ao, A. Wang, G. Lu, L. Kong, Y. Gong, N. Zhao, and X. Zhang, A sandwich tubular scaffold derived from chitosan for blood vessel tissue engineering. J Biomed Mater Res A, 2006. 77(2): p. 277-84.
85. Chung, T.W., J. Yang, T. Akaike, K.Y. Cho, J.W. Nah, S.I. Kim, and C.S. Cho, Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment. Biomaterials, 2002. 23(14): p. 2827-34.
86. Rowley, J.A. and D.J. Mooney, Alginate type and RGD density control myoblast phenotype. J Biomed Mater Res, 2002. 60(2): p. 217-23.
87. Orive, G., R.M. Hernandez, A.R. Gascon, M. Igartua, and J.L. Pedraz, Survival of different cell lines in alginate-agarose microcapsules. Eur J Pharm Sci, 2003. 18(1): p. 23-30.
88. Glicklis, R., L. Shapiro, R. Agbaria, J.C. Merchuk, and S. Cohen, Hepatocyte behavior within three-dimensional porous alginate scaffolds. Biotechnology and Bioengineering, 2000. 67(3): p. 344-353.

89. Dar, A., M. Shachar, J. Leor, and S. Cohen, Cardiac tissue engineering - Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. Biotechnology and Bioengineering, 2002. 80(3): p. 305-312.
90. Masuda, K., R.L. Sah, M.J. Hejna, and E.J.M.A. Thonar, A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (ARC) method. Journal of Orthopaedic Research, 2003. 21(1): p. 139-148.
91. Leor, J., S. Aboulafia-Etzion, A. Dar, L. Shapiro, I.M. Barbash, A. Battler, Y. Granot, and S. Cohen, Bioengineered cardiac grafts - A new approach to repair the infarcted myocardium? Circulation, 2000. 102(19): p. 56-61.
92. Kaur, L., Singh, J. and Liu, Q., Starch - a potential biomaterial for biomedical applications. Nanomaterials and nanosystems for biomedical applications, Springer, 2007: p. 83-98.
93. Pereira, C.S., A.M. Cunha, R.L. Reis, B. Vazquez, and J. San Roman, New starch-based thermoplastic hydrogels for use as bone cements or drug-delivery carriers. Journal of Materials Science-Materials in Medicine, 1998. 9(12): p. 825-833.
94. Gomes, M.E., A.S. Ribeiro, P.B. Malafaya, R.L. Reis, and A.M. Cunha, A new approach based on injection moulding to produce biodegradable starch-based polymeric scaffolds: morphology, mechanical and degradation behaviour. Biomaterials, 2001. 22(9): p. 883-889.
95. Santos, M.I., S. Fuchs, M.E. Gomes, R.E. Unger, R.L. Reis, and C.J. Kirkpatrick, Response of micro- and macrovascular endothelial cells to starch-based fiber meshes for bone tissue engineering. Biomaterials, 2007. 28(2): p. 240-8.
96. Liao, Y.H., S.A. Jones, B. Forbes, G.P. Martin, and M.B. Brown, Hyaluronan: pharmaceutical characterization and drug delivery. Drug Deliv, 2005. 12(6): p. 327-42.
97. Fraser, J.R., T.C. Laurent, and U.B. Laurent, Hyaluronan: its nature, distribution, functions and turnover. J Intern Med, 1997. 242(1): p. 27-33.
98. Laurent, T.C. and J.R.E. Fraser, Hyaluronan. Faseb Journal, 1992. 6(7): p. 2397-2404.
99. West, D.C. and S. Kumar, Hyaluronan and Angiogenesis. Ciba Foundation Symposia, 1989. 143: p. 187-207.
100. Baier Leach, J.a.S., C.E., Hyaluronan. Encyclopedia of Biomaterials and Biomedical Engineering, 2004: p. 779-789.
101. Li, S. and J.J. Henry, Nonthrombogenic approaches to cardiovascular bioengineering. Annu Rev Biomed Eng, 2011. 13: p. 451-75.
102. Kito, H. and T. Matsuda, Biocompatible coatings for luminal and outer surfaces of small-caliber artificial grafts. J Biomed Mater Res, 1996. 30(3): p. 321-30.
103. Xu, X.L., J. Lou, T. Tang, K.W. Ng, J. Zhang, C. Yu, and K. Dai, Evaluation of different scaffolds for BMP-2 genetic orthopedic tissue engineering. J Biomed Mater Res B Appl Biomater, 2005. 75(2): p. 289-303.
104. Radice, M., P. Brun, R. Cortivo, R. Scapinelli, C. Battaliard, and G. Abatangelo, Hyaluronan-based biopolymers as delivery vehicles for bone-marrow-derived mesenchymal progenitors. J Biomed Mater Res, 2000. 50(2): p. 101-9.
105. Aigner, J., J. Tegeler, P. Hutzler, D. Campoccia, A. Pavesio, C. Hammer, E. Kastenbauer, and A. Naumann, Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester. J Biomed Mater Res, 1998. 42(2): p. 172-81.
106. Grant, M.E., From collagen chemistry towards cell therapy - A personal journey. International Journal of Experimental Pathology, 2007. 88(4): p. 203-214.
107. Smith, K. and M.J. Rennie, New approaches and recent results concerning human-tissue collagen synthesis. Current Opinion in Clinical Nutrition and Metabolic Care, 2007. 10(5): p. 582-590.
108. Auger, F.A., M. Rouabhia, F. Goulet, F. Berthod, V. Moulin, and L. Germain, Tissue-engineered human skin substitutes developed from collagen-populated hydrated gels: clinical and fundamental applications. Medical & Biological Engineering & Computing, 1998. 36(6): p. 801-812.
109. Yannas, I.V., J.F. Burke, D.P. Orgill, and E.M. Skrabut, Wound Tissue Can Utilize a Polymeric Template to Synthesize a Functional Extension of Skin. Science, 1982. 215(4529): p. 174-176.
110. Weadock, K.S., E.J. Miller, E.L. Keuffel, and M.G. Dunn, Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions. J Biomed Mater Res, 1996. 32(2): p. 221-6.

111. Taylor, P.M., S.P. Allen, S.A. Dreger, and M.H. Yacoub, Human cardiac valve interstitial cells in collagen sponge: A biological three-dimensional matrix for tissue engineering. Journal of Heart Valve Disease, 2002. 11(3): p. 298-306.
112. Butcher, J.T. and R.M. Nerem, Porcine aortic valve interstitial cells in three-dimensional culture: Comparison of phenotype with aortic smooth muscle cells. Journal of Heart Valve Disease, 2004. 13(3): p. 478-485.
113. Tedder, M.E., J. Liao, B. Weed, C. Stabler, H. Zhang, A. Simionescu, and D.T. Simionescu, Stabilized Collagen Scaffolds for Heart Valve Tissue Engineering. Tissue Engineering Part A, 2009. 15(6): p. 1257-1268.
114. Rothenburger, M., W. Volker, P. Vischer, B. Glasmacher, H.H. Scheld, and M. Deiwick, Ultrastructure of Proteoglycans in tissue-engineered cardiovascular structures. Tissue Engineering, 2002. 8(6): p. 1049-1056.
115. Weinberg, C.B. and E. Bell, A blood vessel model constructed from collagen and cultured vascular cells. Science, 1986. 231(4736): p. 397-400.
116. Ikada, Y. and Y. Tabata, Protein release from gelatin matrices. Adv Drug Deliv Rev, 1998. 31(3): p. 287-301.
117. Tan, H.P. and K.G. Marra, Injectable, Biodegradable Hydrogels for Tissue Engineering Applications. Materials, 2010. 3(3): p. 1746-1767.
118. Sakai, S., K. Hirose, K. Taguchi, Y. Ogushi, and K. Kawakami, An injectable, in situ enzymatically gellable, gelatin derivative for drug delivery and tissue engineering. Biomaterials, 2009. 30(20): p. 3371-3377.
119. Mao, J.S., L.G. Zhao, K. de Yao, Q.X. Shang, G.H. Yang, and Y.L. Cao, Study of novel chitosan-gelatin artificial skin in vitro. Journal of Biomedical Materials Research Part A, 2003. 64A(2): p. 301-308.
120. Li, R.K., Z.Q. Jia, R.D. Weisel, D.A.G. Mickle, A. Choi, and T.M. Yau, Survival and function of bioengineered cardiac grafts. Circulation, 1999. 100(19): p. 63-69.
121. Mosesson, M.W., K.R. Siebenlist, and D.A. Meh, The structure and biological features of fibrinogen and fibrin. Fibrinogen, 2001. 936: p. 11-30.
122. Dietrich, M., J. Heselhaus, J. Wozniak, S. Weinandy, P. Mela, B. Tschoeke, T. Schmitz-Rode, and S. Jockenhoevel, Fibrin-Based Tissue Engineering: Comparison of Different Methods of Autologous Fibrinogen Isolation. Tissue Engineering Part C-Methods, 2013. 19(3): p. 216-226.
123. Mol, A., M.I. van Lieshout, C.G.D. Veen, S. Neuenschwander, S.P. Hoerstrup, F.P.T. Baaijens, and C.V.C. Bouten, Fibrin as a cell carrier in cardiovascular tissue engineering applications. Biomaterials, 2005. 26(16): p. 3113-3121.
124. Cho, S.W., I. Kim, S.H. Kim, J.W. Rhie, C.Y. Choi, and B.S. Kim, Enhancement of adipose tissue formation by implantation of adipogenic-differentiated preadipocytes. Biochemical and Biophysical Research Communications, 2006. 345(2): p. 588-594.
125. Eyrich, D., F. Brandl, B. Appel, H. Wiese, G. Maier, M. Wenzel, R. Staudenmaier, A. Goepferich, and T. Blunk, Long-term stable fibrin gels for cartilage engineering. Biomaterials, 2007. 28(1): p. 55-65.
126. Ahmed, T.A.E., E.V. Dare, and M. Hincke, Fibrin: A versatile scaffold for tissue engineering applications. Tissue Engineering Part B-Reviews, 2008. 14(2): p. 199-215.
127. Cummings, C.L., D. Gawlitta, R.M. Nerem, and J.P. Stegemann, Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures. Biomaterials, 2004. 25(17): p. 3699-3706.
128. Long, J.L. and R.T. Tranquillo, Elastic fiber production in cardiovascular tissue-equivalents. Matrix Biology, 2003. 22(4): p. 339-350.
129. Jockenhoevel, S., G. Zund, S.P. Hoerstrup, K. Chalabi, J.S. Sachweh, L. Demircan, B.J. Messmer, and M. Turina, Fibrin gel-advantages of a new scaffold in cardiovascular tissue engineering. European Journal of Cardio-Thoracic Surgery, 2001. 19(4): p. 424-430.
130. Chiu, L.L. and M. Radisic, Scaffolds with covalently immobilized VEGF and Angiopoietin-1 for vascularization of engineered tissues. Biomaterials, 2010. 31(2): p. 226-41.
131. Kang, H.M., M.H. Kalnoski, M. Frederick, and W.L. Chandler, The kinetics of plasmin inhibition by aprotinin in vivo. Thrombosis Research, 2005. 115(4): p. 327-340.
132. Adachi, E., I. Hopkinson, and T. Hayashi, Basement-membrane stromal relationships: interactions between collagen fibrils and the lamina densa. Int Rev Cytol, 1997. 173: p. 73-156.
133. Schmidt, C.E. and J.M. Baier, Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. Biomaterials, 2000. 21(22): p. 2215-31.

134. Gilbert, T.W., T.L. Sellaro, and S.F. Badylak, Decellularization of tissues and organs. Biomaterials, 2006. 27(19): p. 3675-83.
135. Mirsadraee, S., H.E. Wilcox, S.A. Korossis, J.N. Kearney, K.G. Watterson, J. Fisher, and E. Ingham, Development and characterization of an acellular human pericardial matrix for tissue engineering. Tissue Eng, 2006. 12(4): p. 763-73.
136. Mirsadraee, S., H.E. Wilcox, K.G. Watterson, J.N. Kearney, J. Hunt, J. Fisher, and E. Ingham, Biocompatibility of acellular human pericardium. J Surg Res, 2007. 143(2): p. 407-14.
137. Elkins, R.C., P.E. Dawson, S. Goldstein, S.P. Walsh, and K.S. Black, Decellularized human valve allografts. Ann Thorac Surg, 2001. 71(5 Suppl): p. S428-32.
138. Hopkins, R.A., A.L. Jones, L. Wolfinbarger, M.A. Moore, A.A. Bert, and G.K. Lofland, Decellularization reduces calcification while improving both durability and 1-year functional results of pulmonary homograft valves in juvenile sheep. J Thorac Cardiovasc Surg, 2009. 137(4): p. 907-13, 913e1-4.
139. Neuenschwander, S. and S.P. Hoerstrup, Heart valve tissue engineering. Transpl.Immunol., 2004. 12(3-4): p. 359-365.
140. Kasimir, M.T., E. Rieder, G. Seebacher, G. Silberhumer, E. Wolner, G. Weigel, and P. Simon, Comparison of different decellularization procedures of porcine heart valves. Int.J.Artif.Organs, 2003. 26(5): p. 421-427.
141. Spina, M., F. Ortolani, M.A. El, A. Gandaglia, J. Bujan, N. Garcia-Honduvilla, I. Vesely, G. Gerosa, D. Casarotto, L. Petrelli, and M. Marchini, Isolation of intact aortic valve scaffolds for heart-valve bioprostheses: extracellular matrix structure, prevention from calcification, and cell repopulation features. J.Biomed.Mater.Res.A, 2003. 67(4): p. 1338-1350.
142. Grauss, R.W., M.G. Hazekamp, F. Oppenhuizen, C.J. van Munsteren, A.C. Gittenberger-de Groot, and M.C. DeRuiter, Histological evaluation of decellularised porcine aortic valves: matrix changes due to different decellularisation methods. Eur.J.Cardiothorac.Surg., 2005. 27(4): p. 566-571.
143. Gilbert, T.W., T.L. Sellaro, and S.F. Badylak, Decellularization of tissues and organs. Biomaterials, 2006. 27(19): p. 3675-3683.
144. Iop, L., V. Renier, F. Naso, M. Piccoli, A. Bonetti, A. Gandaglia, M. Pozzobon, A. Paolin, F. Ortolani, M. Marchini, M. Spina, C.P. De, S. Sartore, and G. Gerosa, The influence of heart valve leaflet matrix characteristics on the interaction between human mesenchymal stem cells and decellularized scaffolds. Biomaterials, 2009. 30(25): p. 4104-4116.
145. Badylak, S.F., D.O. Freytes, and T.W. Gilbert, Extracellular matrix as a biological scaffold material: Structure and function. Acta Biomater, 2009. 5(1): p. 1-13.
146. Piterina, A.V., A.J. Cloonan, C.L. Meaney, L.M. Davis, A. Callanan, M.T. Walsh, and T.M. McGloughlin, ECM-based materials in cardiovascular applications: Inherent healing potential and augmentation of native regenerative processes. Int J Mol Sci, 2009. 10(10): p. 4375-417.
147. Human, P. and P. Zilla, Characterization of the immune response to valve bioprostheses and its role in primary tissue failure. Ann.Thorac.Surg., 2001. 71(5 Suppl): p. S385-S388.
148. Gilbert, T.W., F.S. JM, and S.F. Badylak, Quantification of DNA in Biologic Scaffold Materials. J.Surg.Res., 2008.
149. Liao, J., E.M. Joyce, and M.S. Sacks, Effects of decellularization on the mechanical and structural properties of the porcine aortic valve leaflet. Biomaterials, 2008. 29(8): p. 1065-1074.
150. Dong, X., X. Wei, W. Yi, C. Gu, X. Kang, Y. Liu, Q. Li, and D. Yi, RGD-modified acellular bovine pericardium as a bioprosthetic scaffold for tissue engineering. J.Mater.Sci.Mater.Med., 2009.
151. Huszar, G., J. Maiocco, and F. Naftolin, Monitoring of collagen and collagen fragments in chromatography of protein mixtures. Anal.Biochem., 1980. 105(2): p. 424-429.
152. Hutmacher, D.W., J.C. Goh, and S.H. Teoh, An introduction to biodegradable materials for tissue engineering applications. Ann.Acad Med.Singapore, 2001. 30(2): p. 183-191.
153. Konduri, S., Y. Xing, J.N. Warnock, Z. He, and A.P. Yoganathan, Normal physiological conditions maintain the biological characteristics of porcine aortic heart valves: an ex vivo organ culture study. Ann.Biomed.Eng, 2005. 33(9): p. 1158-1166.
154. Rabkin, E., M. Aikawa, J.R. Stone, Y. Fukumoto, P. Libby, and F.J. Schoen, Activated interstitial myofibroblasts express catabolic enzymes and mediate matrix remodeling in myxomatous heart valves. Circulation, 2001. 104(21): p. 2525-2532.

155. Rabkin, E., S.P. Hoerstrup, M. Aikawa, J.E.J. Mayer, and F.J. Schoen, Evolution of cell phenotype and extracellular matrix in tissue-engineered heart valves during in-vitro maturation and in-vivo remodeling. J.Heart Valve Dis., 2002. 11(3): p. 308-314.
156. Radisic, M., L. Yang, J. Boublik, R.J. Cohen, R. Langer, L.E. Freed, and G. Vunjak-Novakovic, Medium perfusion enables engineering of compact and contractile cardiac tissue. Am.J.Physiol Heart Circ.Physiol, 2004. 286(2): p. H507-H516.
157. Rand, J.H., L. Badimon, R.E. Gordon, R.R. Uson, and V. Fuster, Distribution of von Willebrand factor in porcine intima varies with blood vessel type and location. Arteriosclerosis, 1987. 7(3): p. 287-291.
158. Rezai, N., T.J. Podor, and B.M. McManus, Bone marrow cells in the repair and modulation of heart and blood vessels: emerging opportunities in native and engineered tissue and biomechanical materials. Artif.Organs, 2004. 28(2): p. 142-151.
159. Roy, A., N.J. Brand, and M.H. Yacoub, Molecular characterization of interstitial cells isolated from human heart valves. J.Heart Valve Dis., 2000. 9(3): p. 459-464.
160. Rubbens, M.P., 2006.
161. Schenke-Layland, K., I. Riemann, F. Opitz, K. Konig, K.J. Halbhuber, and U.A. Stock, Comparative study of cellular and extracellular matrix composition of native and tissue engineered heart valves. Matrix Biol., 2004. 23(2): p. 113-125.
162. Schoen, F.J., S. MD, and A.I. Gotlieb, Pathology of heart valve substitution with mechanical and tissue prostheses, in Cardiovascular Pathology. 2001: Nwe York. p. 629-677.
163. Shinoka, T., C.K. Breuer, R.E. Tanel, G. Zund, T. Miura, P.X. Ma, R. Langer, J.P. Vacanti, and J.E.J. Mayer, Tissue engineering heart valves: valve leaflet replacement study in a lamb model. Ann.Thorac.Surg., 1995. 60(6 Suppl): p. S513-S516.
164. Shinoka, T., P.X. Ma, D. Shum-Tim, C.K. Breuer, R.A. Cusick, G. Zund, R. Langer, J.P. Vacanti, and J.E.J. Mayer, Tissue-engineered heart valves. Autologous valve leaflet replacement study in a lamb model. Circulation, 1996. 94(9 Suppl): p. II164-II168.
165. Sodian, R., S.P. Hoerstrup, J.S. Sperling, S.H. Daebritz, D.P. Martin, F.J. Schoen, J.P. Vacanti, and J.E.J. Mayer, Tissue engineering of heart valves: in vitro experiences. Ann.Thorac.Surg., 2000. 70(1): p. 140-144.
166. Balguid, A., A. Mol, M.H. van Marion, R.A. Bank, C.V. Bouten, and F.P. Baaijens, Tailoring fiber diameter in electrospun poly(epsilon-caprolactone) scaffolds for optimal cellular infiltration in cardiovascular tissue engineering. Tissue Eng Part A, 2009. 15(2): p. 437-44.
167. Li, W.J., J.A. Cooper, Jr., R.L. Mauck, and R.S. Tuan, Fabrication and characterization of six electrospun poly(alpha-hydroxy ester)-based fibrous scaffolds for tissue engineering applications. Acta Biomater, 2006. 2(4): p. 377-85.
168. Singh, S., B.M. Wu, and J.C.Y. Dunn, The enhancement of VEGF-mediated angiogenesis by polycaprolactone scaffolds with surface cross-linked heparin. Biomaterials, 2011. 32(8): p. 2059-2069.
169. Lam, M.T. and J.C. Wu, Biomaterial applications in cardiovascular tissue repair and regeneration. Expert Rev Cardiovasc Ther, 2012. 10(8): p. 1039-49.
170. Qi, J., L. Chi, J. Wang, R. Sumanasinghe, M. Wall, M. Tsuzaki, and A.J. Banes, Modulation of collagen gel compaction by extracellular ATP is MAPK and NF-kappaB pathways dependent. Exp Cell Res, 2009. 315(11): p. 1990-2000.
171. Fernandez, P. and A.R. Bausch, The compaction of gels by cells: a case of collective mechanical activity. Integr Biol (Camb), 2009. 1(3): p. 252-9.
172. Zhou, M., C. Liu, Z. Wei, Z. Liu, X. Jiang, T. Qiao, and F. Ran, [Constructing a small-diameter decellularized vascular graft pre-loaded with bFGF]. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi, 2008. 22(3): p. 370-5.
173. Girton, T.S., T.R. Oegema, E.D. Grassl, B.C. Isenberg, and R.T. Tranquillo, Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation. J Biomech Eng, 2000. 122(3): p. 216-23.
174. Miwa, H., T. Matsuda, and F. Iida, Development of a hierarchically structured hybrid vascular graft biomimicking natural arteries. ASAIO J, 1993. 39(3): p. M273-7.
175. Berglund, J.D., R.M. Nerem, and A. Sambanis, Incorporation of intact elastin scaffolds in tissue-engineered collagen-based vascular grafts. Tissue Eng, 2004. 10(9-10): p. 1526-35.
176. Berglund, J.D., M.M. Mohseni, R.M. Nerem, and A. Sambanis, A biological hybrid model for collagen-based tissue engineered vascular constructs. Biomaterials, 2003. 24(7): p. 1241-54.

177. Seliktar, D., R.A. Black, R.P. Vito, and R.M. Nerem, Dynamic mechanical conditioning of collagen-gel blood vessel constructs induces remodeling in vitro. Ann Biomed Eng, 2000. 28(4): p. 351-62.
178. L'Heureux, N., S. Paquet, R. Labbe, L. Germain, and F.A. Auger, A completely biological tissue-engineered human blood vessel. FASEB J, 1998. 12(1): p. 47-56.
179. Dahl, S.L., A.P. Kypson, J.H. Lawson, J.L. Blum, J.T. Strader, Y. Li, R.J. Manson, W.E. Tente, L. DiBernardo, M.T. Hensley, R. Carter, T.P. Williams, H.L. Prichard, M.S. Dey, K.G. Begelman, and L.E. Niklason, Readily available tissue-engineered vascular grafts. Sci Transl Med, 2011. 3(68): p. 68ra9.
180. Gui, L. and L.E. Niklason, Vascular Tissue Engineering: Building Perfusable Vasculature for Implantation. Curr Opin Chem Eng, 2014. 3: p. 68-74.
181. Shin'oka, T., Y. Imai, and Y. Ikada, Transplantation of a tissue-engineered pulmonary artery. N Engl J Med, 2001. 344(7): p. 532-3.
182. Hibino, N., E. McGillicuddy, G. Matsumura, Y. Ichihara, Y. Naito, C. Breuer, and T. Shinoka, Late-term results of tissue-engineered vascular grafts in humans. Journal of Thoracic and Cardiovascular Surgery, 2010. 139(2): p. 431-U233.
183. Shin'oka, T., G. Matsumura, N. Hibino, Y. Naito, M. Watanabe, T. Konuma, T. Sakamoto, M. Nagatsu, and H. Kurosawa, Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. Journal of Thoracic and Cardiovascular Surgery, 2005. 129(6): p. 1330-1338.
184. Li, D.Y., G. Faury, D.G. Taylor, E.C. Davis, W.A. Boyle, R.P. Mecham, P. Stenzel, B. Boak, and M.T. Keating, Novel arterial pathology in mice and humans hemizygous for elastin. J Clin Invest, 1998. 102(10): p. 1783-7.
185. Cines, D.B., E.S. Pollak, C.A. Buck, J. Loscalzo, G.A. Zimmerman, R.P. McEver, J.S. Pober, T.M. Wick, B.A. Konkle, B.S. Schwartz, E.S. Barnathan, K.R. McCrae, B.A. Hug, A.M. Schmidt, and D.M. Stern, Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood, 1998. 91(10): p. 3527-61.
186. Cho, S.W., S.H. Lim, I.K. Kim, Y.S. Hong, S.S. Kim, K.J. Yoo, H.Y. Park, Y. Jang, B.C. Chang, C.Y. Choi, K.C. Hwang, and B.S. Kim, Small-diameter blood vessels engineered with bone marrow-derived cells. Ann Surg, 2005. 241(3): p. 506-15.
187. Hibino, N., T. Shin'oka, G. Matsumura, Y. Ikada, and H. Kurosawa, The tissue-engineered vascular graft using bone marrow without culture. J Thorac Cardiovasc Surg, 2005. 129(5): p. 1064-70.
188. Matsumura, G., N. Hibino, Y. Ikada, H. Kurosawa, and T. Shin'oka, Successful application of tissue engineered vascular autografts: clinical experience. Biomaterials, 2003. 24(13): p. 2303-8.
189. Shin'oka, T., G. Matsumura, N. Hibino, Y. Naito, M. Watanabe, T. Konuma, T. Sakamoto, M. Nagatsu, and H. Kurosawa, Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells. J Thorac Cardiovasc Surg, 2005. 129(6): p. 1330-8.
190. Wang, C., L. Cen, S. Yin, Q. Liu, W. Liu, Y. Cao, and L. Cui, A small diameter elastic blood vessel wall prepared under pulsatile conditions from polyglycolic acid mesh and smooth muscle cells differentiated from adipose-derived stem cells. Biomaterials, 2010. 31(4): p. 621-30.
191. Visscher, G.d., H. Blockx, B. Meuris, H.v. Oosterwyck, E. Verbeken, M.C. Herregods, and W. Flameng, Functional and biomechanical evaluation of a completely recellularized stentless pulmonary bioprosthesis in sheep. J.Thorac.Cardiovasc.Surg., 2008. 135(2): p. 395-404.
192. Tedder, M.E., A. Simionescu, J. Chen, J. Liao, and D.T. Simionescu, Assembly and testing of stem cell-seeded layered collagen constructs for heart valve tissue engineering. Tissue Eng Part A, 2011. 17(1-2): p. 25-36.
193. Badylak, S.F. and T.W. Gilbert, Immune response to biologic scaffold materials. Semin Immunol, 2008. 20(2): p. 109-16.
194. Byrne, G.W. and C.G. McGregor, Cardiac xenotransplantation: progress and challenges. Curr Opin Organ Transplant, 2012. 17(2): p. 148-54.
195. Cebotari, S., I. Tudorache, A. Ciubotaru, D. Boethig, S. Sarikouch, A. Goerler, A. Lichtenberg, E. Cheptanaru, S. Barnaciuc, A. Cazacu, O. Maliga, O. Repin, L. Maniuc, T. Breymann, and A. Haverich, Use of fresh decellularized allografts for pulmonary valve replacement may reduce the reoperation rate in children and young adults: early report. Circulation, 2011. 124(11 Suppl): p. S115-S123.
196. Dohmen, P.M., A. Lembcke, S. Holinski, A. Pruss, and W. Konertz, Ten years of clinical results with a tissue-engineered pulmonary valve. Ann.Thorac.Surg., 2011. 92(4): p. 1308-1314.

197. O'brien, M.F., S. Goldstein, S. Walsh, K.S. Black, R. Elkins, and D. Clarke, The SynerGraft valve: a new acellular (nonglutaraldehyde-fixed) tissue heart valve for autologous recellularization first experimental studies before clinical implantation. Semin.Thorac.Cardiovasc.Surg., 1999. 11(4 Suppl 1): p. 194-200.
198. Elkins, R.C., S. Goldstein, C.W. Hewitt, S.P. Walsh, P.E. Dawson, J.D. Ollerenshaw, K.S. Black, D.R. Clarke, and M.F. O'brien, Recellularization of heart valve grafts by a process of adaptive remodeling. Semin.Thorac.Cardiovasc.Surg., 2001. 13(4 Suppl 1): p. 87-92.
199. Hopkins, R.A., A.L. Jones, L. Wolfinbarger, M.A. Moore, A.A. Bert, and G.K. Lofland, Decellularization reduces calcification while improving both durability and 1-year functional results of pulmonary homograft valves in juvenile sheep. J.Thorac.Cardiovasc.Surg., 2009. 137(4): p. 907-4.
200. Konertz, W., E. Angeli, G. Tarusinov, T. Christ, J. Kroll, P.M. Dohmen, O. Krogmann, B. Franzbach, C. Pace Napoleone, and G. Gargiulo, Right ventricular outflow tract reconstruction with decellularized porcine xenografts in patients with congenital heart disease. J.Heart Valve Dis., 2011. 20(3): p. 341-347.
201. Sayk, F., I. Bos, U. Schubert, T. Wedel, and H.H. Sievers, Histopathologic findings in a novel decellularized pulmonary homograft: an autopsy study. Ann.Thorac.Surg., 2005. 79(5): p. 1755-1758.
202. Dohmen, P.M., S. Hauptmann, A. Terytze, and W.F. Konertz, In-vivo repopularization of a tissue-engineered heart valve in a human subject. J.Heart Valve Dis., 2007. 16(4): p. 447-449.
203. Miller, D.V., W.D. Edwards, and K.J. Zehr, Endothelial and smooth muscle cell populations in a decellularized cryopreserved aortic homograft (SynerGraft) 2 years after implantation. J.Thorac.Cardiovasc.Surg., 2006. 132(1): p. 175-176.
204. Simon, P., M.T. Kasimir, G. Seebacher, G. Weigel, R. Ullrich, U. Salzer-Muhar, E. Rieder, and E. Wolner, Early failure of the tissue engineered porcine heart valve SYNERGRAFT in pediatric patients. Eur.J.Cardiothorac.Surg., 2003. 23(6): p. 1002-1006.
205. Hoerstrup, S.P., R. Sodian, S. Daebritz, J. Wang, E.A. Bacha, D.P. Martin, A.M. Moran, K.J. Guleserian, J.S. Sperling, S. Kaushal, J.P. Vacanti, F.J. Schoen, and J.E.J. Mayer, Functional living trileaflet heart valves grown in vitro. Circulation, 2000. 102(19 Suppl 3): p. III44-III49.
206. Steinhoff, G., U. Stock, N. Karim, H. Mertsching, A. Timke, R.R. Meliss, K. Pethig, A. Haverich, and A. Bader, Tissue engineering of pulmonary heart valves on allogenic acellular matrix conduits: in vivo restoration of valve tissue. Circulation, 2000. 102(19 Suppl 3): p. III50-III55.
207. Sodian, R., S.P. Hoerstrup, J.S. Sperling, S. Daebritz, D.P. Martin, A.M. Moran, B.S. Kim, F.J. Schoen, J.P. Vacanti, and J.E. Mayer, Jr., Early in vivo experience with tissue-engineered trileaflet heart valves. Circulation, 2000. 102(19 Suppl 3): p. III22-III29.
208. Hoerstrup, S.P., A. Kadner, S. Melnitchouk, A. Trojan, K. Eid, J. Tracy, R. Sodian, J.F. Visjager, S.A. Kolb, J. Grunenfelder, G. Zund, and M.I. Turina, Tissue engineering of functional trileaflet heart valves from human marrow stromal cells. Circulation, 2002. 106(12 Suppl 1): p. I143-I150.
209. Sutherland, F.W.H., T.E. Perry, Y. Yu, M.C. Sherwood, E. Rabkin, Y. Masuda, G.A. Garcia, D.L. McLellan, G.C.J. Engelmayr, M.S. Sacks, F.J. Schoen, and J.E.J. Mayer, From stem cells to viable autologous semilunar heart valve. Circulation, 2005. 111(21): p. 2783-2791.
210. Mol, A., M.C. Rutten, N.J. Driessen, C.V. Bouten, G. Zund, F.P. Baaijens, and S.P. Hoerstrup, Autologous human tissue-engineered heart valves: prospects for systemic application. Circulation, 2006. 114(1 Suppl): p. I152-I158.
211. Robinson, P.S., S.L. Johnson, M.C. Evans, V.H. Barocas, and R.T. Tranquillo, Functional tissue-engineered valves from cell-remodeled fibrin with commissural alignment of cell-produced collagen. Tissue Eng Part A, 2008. 14(1): p. 83-95.
212. Flanagan, T.C., J.S. Sachweh, J. Frese, H. Schnoring, N. Gronloh, S. Koch, R.H. Tolba, T. Schmitz-Rode, and S. Jockenhoevel, In vivo remodeling and structural characterization of fibrin-based tissue-engineered heart valves in the adult sheep model. Tissue Eng Part A, 2009. 15(10): p. 2965-2976.
213. Gottlieb, D., T. Kunal, S. Emani, E. Aikawa, D.W. Brown, A.J. Powell, A. Nedder, G.C.J. Engelmayr, J.M. Melero-Martin, M.S. Sacks, and J.E.J. Mayer, In vivo monitoring of function of autologous engineered pulmonary valve. J.Thorac.Cardiovasc.Surg., 2010. 139(3): p. 723-731.
214. Schmidt, D., P.E. Dijkman, A. Driessen-Mol, R. Stenger, C. Mariani, A. Puolakka, M. Rissanen, T. Deichmann, B. Odermatt, B. Weber, M.Y. Emmert, G. Zund, F.P.T. Baaijens, and S.P. Hoerstrup, Minimally-invasive implantation of living tissue engineered heart valves: a comprehensive approach from autologous vascular cells to stem cells. J.Am.Coll.Cardiol., 2010. 56(6): p. 510-520.

215. Syedain, Z.H., M.T. Lahti, S.L. Johnson, P.S. Robinson, G.R. Ruth, R.W. Bianco, and R.T. Tranquillo, Implantation of a Tissue-engineered Heart Valve from Human Fibroblasts Exhibiting Short Term Function in the Sheep Pulmonary Artery. Cardiovascular Engineering and Technology, 2011. 2(2): p. 101-102.
216. Syedain, Z.H. and R.T. Tranquillo, Controlled cyclic stretch bioreactor for tissue-engineered heart valves. Biomaterials, 2009. 30(25): p. 4078-4084.
217. Schmidt, D., J. Achermann, B. Odermatt, C. Breymann, A. Mol, M. Genoni, G. Zund, and S.P. Hoerstrup, Prenatally fabricated autologous human living heart valves based on amniotic fluid derived progenitor cells as single cell source. Circulation, 2007. 116(11 Suppl): p. I64-I70.
218. Sales, V.L., B.A. Mettler, G.C. Engelmayr, E. Aikawa, J. Bischoff, D.P. Martin, A. Exarhopoulos, M. Moses, F.J. Schoen, M. Sacks, and J. Mayer, Endothelial Progenitor Cells as a Sole Source for Ex vivo Seeding of Tissue-engineered Heart Valves. Tissue Eng Part A, 2009.
219. Sodian, R., P. Schaefermeier, S. begg-Zips, W.M. Kuebler, M. Shakibaei, S. Daebritz, J. Ziegelmueller, C. Schmitz, and B. Reichart, Use of human umbilical cord blood-derived progenitor cells for tissue-engineered heart valves. Ann.Thorac.Surg., 2010. 89(3): p. 819-828.
220. Gui, L., S.A. Chan, C.K. Breuer, and L.E. Niklason, Novel utilization of serum in tissue decellularization. Tissue Eng Part C.Methods, 2010. 16(2): p. 173-184.
221. Brown, B.N., J.E. Valentin, A.M. Stewart-Akers, G.P. McCabe, and S.F. Badylak, Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. Biomaterials, 2009. 30(8): p. 1482-1491.
222. Rieder, E., M.T. Kasimir, G. Silberhumer, G. Seebacher, E. Wolner, P. Simon, and G. Weigel, Decellularization protocols of porcine heart valves differ importantly in efficiency of cell removal and susceptibility of the matrix to recellularization with human vascular cells. J.Thorac.Cardiovasc.Surg., 2004. 127(2): p. 399-405.
223. Vincentelli, A., F. Wautot, F. Juthier, O. Fouquet, D. Corseaux, S. Marechaux, T.T. Le, O. Fabre, S. Susen, B.E. Van, F. Mouquet, C. Decoene, A. Prat, and B. Jude, In vivo autologous recellularization of a tissue-engineered heart valve: are bone marrow mesenchymal stem cells the best candidates? J.Thorac.Cardiovasc.Surg., 2007. 134(2): p. 424-432.
224. Perry, T.E., S. Kaushal, F.W. Sutherland, K.J. Guleserian, J. Bischoff, M. Sacks, and J.E. Mayer, Thoracic Surgery Directors Association Award. Bone marrow as a cell source for tissue engineering heart valves. Ann.Thorac.Surg., 2003. 75(3): p. 761-767.
225. Latif, N., P. Sarathchandra, P.S. Thomas, J. Antoniw, P. Batten, A.H. Chester, P.M. Taylor, and M.H. Yacoub, Characterization of structural and signaling molecules by human valve interstitial cells and comparison to human mesenchymal stem cells. J.Heart Valve Dis., 2007. 16(1): p. 56-66.
226. Hashi, C.K., Y. Zhu, G.Y. Yang, W.L. Young, B.S. Hsiao, K. Wang, B. Chu, and S. Li, Antithrombogenic property of bone marrow mesenchymal stem cells in nanofibrous vascular grafts. Proceedings of the National Academy of Sciences, 2007. 104(29): p. 11915-11920.
227. Uccelli, A., L. Moretta, and V. Pistoia, Immunoregulatory function of mesenchymal stem cells. Eur.J.Immunol., 2006. 36(10): p. 2566-2573.
228. Pittenger, M.F. and B.J. Martin, Mesenchymal stem cells and their potential as cardiac therapeutics. Circ.Res., 2004. 95(1): p. 9-20.
229. Mirza, A., J.M. Hyvelin, G.Y. Rochefort, P. Lermusiaux, D. Antier, B. Awede, P. Bonnet, J. Domenech, and V. Eder, Undifferentiated mesenchymal stem cells seeded on a vascular prosthesis contribute to the restoration of a physiologic vascular wall. J.Vasc.Surg., 2008. 47(6): p. 1313-1321.
230. Weber, B., S. J, E. MY, G. J, V. R, B. M, B. M, K. J, F. T, S. R, B. L, B. C, A. I, W. P, Z. G, F. V, Z. P, and H. SP, Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates. Eur.Heart J., 2011.
231. Roh, J.D., R. Sawh-Martinez, M.P. Brennan, S.M. Jay, L. Devine, D.A. Rao, T. Yi, T.L. Mirensky, A. Nalbandian, B. Udelsman, N. Hibino, T. Shinoka, W.M. Saltzman, E. Snyder, T.R. Kyriakides, J.S. Pober, and C.K. Breuer, Tissue-engineered vascular grafts transform into mature blood vessels via an inflammation-mediated process of vascular remodeling. Proc.Natl.Acad.Sci.U.S.A, 2010. 107(10): p. 4669-4674.
232. Shinoka, T. and C. Breuer, Tissue-engineered blood vessels in pediatric cardiac surgery. Yale J Biol Med, 2008. 81(4): p. 161-6.

233. L'Heureux, N., T.N. McAllister, and L.M. de la Fuente, Tissue-engineered blood vessel for adult arterial revascularization. N Engl J Med, 2007. 357(14): p. 1451-3.
234. Cebotari, S., I. Tudorache, A. Ciubotaru, D. Boethig, S. Sarikouch, A. Goerler, A. Lichtenberg, E. Cheptanaru, S. Barnaciuc, A. Cazacu, O. Maliga, O. Repin, L. Maniuc, T. Breymann, and A. Haverich, Use of fresh decellularized allografts for pulmonary valve replacement may reduce the reoperation rate in children and young adults: early report. Circulation, 2011. 124(11 Suppl): p. S115-23.
235. Barocas, V.H., T.S. Girton, and R.T. Tranquillo, Engineered alignment in media equivalents: magnetic prealignment and mandrel compaction. J.Biomech.Eng, 1998. 120(5): p. 660-666.
236. Shi, Y. and I. Vesely, Fabrication of mitral valve chordae by directed collagen gel shrinkage. Tissue Eng, 2003. 9(6): p. 1233-1242.
237. Mol, A., N.J.B. Driessen, M.C.M. Rutten, S.P. Hoerstrup, C.V.C. Bouten, and F.P.T. Baaijens, Tissue engineering of human heart valve leaflets: a novel bioreactor for a strain-based conditioning approach. Ann.Biomed.Eng, 2005. 33(12): p. 1778-1788.
238. Neidert, M.R. and R.T. Tranquillo, Tissue-engineered valves with commissural alignment. Tissue Eng, 2006. 12(4): p. 891-903.
239. Vlimmeren, M.A.v., A. Driessen-Mol, C.W.J. Oomens, and F.P.T. Baaijens, An In Vitro Model System to Quantify Stress Generation, Compaction, and Retraction in Engineered Heart Valve Tissue. Tissue Eng Part C.Methods, 2011.
240. Driessen-Mol, A., M.Y. Emmert, P.E. Dijkman, L. Frese, B. Sanders, B. Weber, N. Cesarovic, M. Sidler, J. Leenders, R. Jenni, J. Grunenfelder, V. Falk, F.P. Baaijens, and S.P. Hoerstrup, Transcatheter implantation of homologous "off-the-shelf" tissue engineered heart valves with self-repair capacity: long term functionality and rapid in vivo remodeling in sheep. J Am Coll Cardiol, 2013.
241. Lutter, G., A. Metzner, T. Jahnke, R. Bombien, J. Boldt, K. Iino, J. Cremer, and U.A. Stock, Percutaneous tissue-engineered pulmonary valved stent implantation. Ann.Thorac.Surg., 2010. 89(1): p. 259-263.
242. Dijkman, P.E., A. Driessen-Mol, L.M. de Heer, J. Kluin, L.A. van Herwerden, B. Odermatt, F.P.T. Baaijens, and S.P. Hoerstrup, Trans-Apical versus Surgical Implantation of Autologous Ovine Tissue-Engineered Heart Valves. Journal of Heart Valve Disease, 2012. 21(5): p. 670-678.
243. Dohmen, P.M., A. Lembcke, S. Holinski, D. Kivelitz, J.P. Braun, A. Pruss, and W. Konertz, Mid-term clinical results using a tissue-engineered pulmonary valve to reconstruct the right ventricular outflow tract during the Ross procedure. Ann.Thorac.Surg., 2007. 84(3): p. 729-736.
244. Hiemann, N.E., M. Mani, M. Huebler, R. Meyer, R. Hetzer, R. Thieme, and C. Bethge, Complete destruction of a tissue-engineered porcine xenograft in pulmonary valve position after the Ross procedure. J.Thorac.Cardiovasc.Surg., 2010. 139(4): p. e67-e68.
245. Woodell, J.E., M. LaBerge, E.M. Langan, and R.H. Hilderman, In vitro strain-induced endothelial cell dysfunction determined by DNA synthesis. Proc.Inst.Mech.Eng [H.], 2003. 217(1): p. 13-20.
246. Ziegler, T., R.W. Alexander, and R.M. Nerem, An endothelial cell-smooth muscle cell co-culture model for use in the investigation of flow effects on vascular biology. Ann.Biomed.Eng, 1995. 23(3): p. 216-225.
247. Zund, G., C.K. Breuer, T. Shinoka, P.X. Ma, R. Langer, J.E. Mayer, and J.P. Vacanti, The in vitro construction of a tissue engineered bioprosthetic heart valve. Eur.J.Cardiothorac.Surg., 1997. 11(3): p. 493-497.
248. Deasy, B.M., Y. Li, and J. Huard, Tissue engineering with muscle-derived stem cells. Curr.Opin.Biotechnol., 2004. 15(5): p. 419-423.
249. Hopkins, R.A., Tissue engineering of heart valves: decellularized valve scaffolds. Circulation, 2005. 111(21): p. 2712-2714.
250. Kallenbach, K., S. Sorrentino, H. Mertsching, S. Kostin, K. Pethig, M.A. Haverich, and S. Cebotari, A Novel Small Animal Model for Accelerated Investigation of Tissue Engineered Aortic Valved Conduits. Tissue Eng Part C.Methods, 2009.
251. Ponce, M.S., V.S. Martinez, A.W. McIntosh, J. Wang, N.G. Gantxegui, O. Schein, and J. Elisseeff, Decellularization of bovine corneas for tissue engineering applications. Acta Biomater., 2009. 5(6): p. 1839-1847.
252. Shi, Y., R. Iyer, A. Soundararajan, D. Dobkin, and I. Vesely, Collagen-based Tissue Engineering as Applied to Heart Valves. Conf.Proc.IEEE Eng Med.Biol.Soc., 2005. 5: p. 4912-4915.

253. Rieder, E., G. Seebacher, M.T. Kasimir, E. Eichmair, B. Winter, B. Dekan, E. Wolner, P. Simon, and G. Weigel, Tissue engineering of heart valves: decellularized porcine and human valve scaffolds differ importantly in residual potential to attract monocytic cells. Circulation, 2005. 111(21): p. 2792-2797.
254. Kasimir, M.T., E. Rieder, G. Seebacher, A. Nigisch, B. Dekan, E. Wolner, G. Weigel, and P. Simon, Decellularization does not eliminate thrombogenicity and inflammatory stimulation in tissue-engineered porcine heart valves. J.Heart Valve Dis., 2006. 15(2): p. 278-286.
255. Dohmen, P.M., A. Lembcke, H. Hotz, D. Kivelitz, and W.F. Konertz, Ross operation with a tissue-engineered heart valve. Ann.Thorac.Surg., 2002. 74(5): p. 1438-1442.
256. Mills, C.D., K. Kincaid, J.M. Alt, M.J. Heilman, and A.M. Hill, M-1/M-2 macrophages and the Th1/Th2 paradigm. J.Immunol., 2000. 164(12): p. 6166-6173.
257. Erdbrugger, W., W. Konertz, P.M. Dohmen, S. Posner, H. Ellerbrok, O.E. Brodde, H. Robenek, D. Modersohn, A. Pruss, S. Holinski, M. Stein-Konertz, and G. Pauli, Decellularized xenogenic heart valves reveal remodeling and growth potential in vivo. Tissue Eng, 2006. 12(8): p. 2059-2068.
258. Stock, U.A., M. Nagashima, P.N. Khalil, G.D. Nollert, T. Herden, J.S. Sperling, A. Moran, J. Lien, D.P. Martin, F.J. Schoen, J.P. Vacanti, and J.E.J. Mayer, Tissue-engineered valved conduits in the pulmonary circulation. J.Thorac.Cardiovasc.Surg., 2000. 119(4 Pt 1): p. 732-740.
259. Schmidt, D., A. Mol, B. Odermatt, S. Neuenschwander, C. Breymann, M. Gossi, M. Genoni, G. Zund, and S.P. Hoerstrup, Engineering of biologically active living heart valve leaflets using human umbilical cord-derived progenitor cells. Tissue Eng, 2006. 12(11): p. 3223-3232.
260. Emmert, M.Y., B. Weber, L. Behr, T. Frauenfelder, C.E. Brokopp, J. Grunenfelder, V. Falk, and S.P. Hoerstrup, Transapical aortic implantation of autologous marrow stromal cell-based tissue-engineered heart valves first experiences in the systemic circulation. JACC.Cardiovasc.Interv., 2011. 4(7): p. 822-823.
261. Del Gaudio, C., A. Bianco, and M. Grigioni, Electrospun bioresorbable trileaflet heart valve prosthesis for tissue engineering: in vitro functional assessment of a pulmonary cardiac valve design. Ann.Ist.Super.Sanita, 2008. 44(2): p. 178-186.
262. Del Gaudio, C., M. Grigioni, A. Bianco, and G. De Angelis, Electrospun bioresorbable heart valve scaffold for tissue engineering. Int.J.Artif.Organs, 2008. 31(1): p. 68-75.
263. Lieshout, M.I.v., C.M. Vaz, M.C.M. Rutten, G.W.M. Peters, and F.P.T. Baaijens, Electrospinning versus knitting: two scaffolds for tissue engineering of the aortic valve. J.Biomater.Sci.Polym.Ed, 2006. 17(1-2): p. 77-89.
264. Jockenhoevel, S., G. Zund, S.P. Hoerstrup, K. Chalabi, J.S. Sachweh, L. Demircan, B.J. Messmer, and M. Turina, Fibrin gel -- advantages of a new scaffold in cardiovascular tissue engineering. Eur.J.Cardiothorac.Surg., 2001. 19(4): p. 424-430.
265. Cebotari, S., A. Lichtenberg, I. Tudorache, A. Hilfiker, H. Mertsching, R. Leyh, T. Breymann, K. Kallenbach, L. Maniuc, A. Batrinac, O. Repin, O. Maliga, A. Ciubotaru, and A. Haverich, Clinical application of tissue engineered human heart valves using autologous progenitor cells. Circulation, 2006. 114(1 Suppl): p. I132-I137.
266. Zehr, K.J., M. Yagubyan, H.M. Connolly, S.M. Nelson, and H.V. Schaff, Aortic root replacement with a novel decellularized cryopreserved aortic homograft: postoperative immunoreactivity and early results. J.Thorac.Cardiovasc.Surg., 2005. 130(4): p. 1010-1015.
267. Dohmen, P.M., C.F. da, S. Yoshi, S.V. Lopes, F.P. da Souza, R. Vilani, A.F. Wouk, C.M. da, and W. Konertz, Histological evaluation of tissue-engineered heart valves implanted in the juvenile sheep model: is there a need for in-vitro seeding? J.Heart Valve Dis., 2006. 15(6): p. 823-829.
268. Lichtenberg, A., I. Tudorache, S. Cebotari, M. Suprunov, G. Tudorache, H. Goerler, J.K. Park, D. Hilfiker-Kleiner, S. Ringes-Lichtenberg, M. Karck, G. Brandes, A. Hilfiker, and A. Haverich, Preclinical testing of tissue-engineered heart valves re-endothelialized under simulated physiological conditions. Circulation, 2006. 114(1 Suppl): p. I559-I565.
269. Lichtenberg, A., S. Cebotari, I. Tudorache, G. Sturz, M. Winterhalter, A. Hilfiker, and A. Haverich, Flow-dependent re-endothelialization of tissue-engineered heart valves. J.Heart Valve Dis., 2006. 15(2): p. 287-293.
270. Baraki, H., I. Tudorache, M. Braun, K. Hoffler, A. Gorler, A. Lichtenberg, C. Bara, A. Calistru, G. Brandes, M. Hewicker-Trautwein, A. Hilfiker, A. Haverich, and S. Cebotari, Orthotopic replacement of the aortic valve with decellularized allograft in a sheep model. Biomaterials, 2009. 30(31): p. 6240-6246.

271. Stock, U.A., I. Degenkolbe, T. Attmann, K. Schenke-Layland, S. Freitag, and G. Lutter, Prevention of device-related tissue damage during percutaneous deployment of tissue-engineered heart valves. J.Thorac.Cardiovasc.Surg., 2006. 131(6): p. 1323-1330.
272. Metzner, A., U.A. Stock, K. Iino, G. Fischer, T. Huemme, J. Boldt, J.H. Braesen, B. Bein, J. Renner, J. Cremer, and G. Lutter, Percutaneous pulmonary valve replacement: autologous tissue-engineered valved stents. Cardiovasc.Res., 2010. 88(3): p. 453-461.
273. Bader, A., T. Schilling, O.E. Teebken, G. Brandes, T. Herden, G. Steinhoff, and A. Haverich, Tissue engineering of heart valves--human endothelial cell seeding of detergent acellularized porcine valves. Eur.J.Cardiothorac.Surg., 1998. 14(3): p. 279-284.
274. Knight, R.L., C. Booth, H.E. Wilcox, J. Fisher, and E. Ingham, Tissue engineering of cardiac valves: re-seeding of acellular porcine aortic valve matrices with human mesenchymal progenitor cells. J.Heart Valve Dis., 2005. 14(6): p. 806-813.
275. Zeltinger, J., L.K. Landeen, H.G. Alexander, I.D. Kidd, and B. Sibanda, Development and characterization of tissue-engineered aortic valves. Tissue Eng, 2001. 7(1): p. 9-22.

Chapter 2

Comparative Analysis of Poly-glycolic Acid-based Hybrid Polymer Starter Matrices for In-vitro Tissue Engineering

Melanie Generali[1*], Debora Kehl[1*], Andrew K. Capulli[2], Kevin K. Parker[2], Simon P. Hoerstrup[1,3,4°] and Benedikt Weber[1,3,4°]

* These authors contributed equally
° These senior authors contributed equally

[1] Institute for Regenerative Medicine (IREM), Center for Therapy Development and Good Manufacturing Practice, University of Zurich, Zurich, Switzerland
[2] Disease Biophysics Group. Wyss Institute for Biologically Inspired Engineering, John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, USA
[3] Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich, Zurich, Switzerland
[4] Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

Colloid and Surfaces B: Biointerfaces (published 2017)

2.1 Introduction

During the last years, the interdisciplinary field of tissue engineering (TE) has emerged as a platform for the development of biological substitutes. The overall goal is the repair and/or regeneration of tissues or organs to resolve major health related concerns in humans. Multiple disciplines, such as cell biology, biomaterial research and biomedical engineering have contributed to the advances of tissue engineering technologies. Any tissue engineering approach is composed of three major components: (1) cells, (2) biocompatible scaffolds, and (3) suitable biochemical (e.g. growth factors) and physical (e.g. cyclic mechanical loading) stimuli supporting tissue formation in vitro and in situ [1]. While the engineered living substitute develops, the biocompatible scaffold should degrade without leaving remnants in the body, requiring a so-called biodegradable starter matrix (scaffold).

A variety of synthetic biodegradable polymers has been investigated as TE scaffold materials, though the main disadvantage of these materials is their lack of functional groups [2, 3]. This results in limited capacity to combine with bioactive elements to reinforce their cell affinity [3]. In general, functional synthetic polymers have unsaturated bonds [4], or functional groups such as hydroxyl [5], carboxyl [6], and amide [7], through which functional biomaterials can be chemically modified by biomolecules to improve their bioactivity [5]. Nonetheless, synthetic polymers have been extensively used for TE, given their high durability, flexibility, and mechanical strength [8]. In addition, production conditions of synthetic polymers can be tightly controlled, hence making mechanical and physical properties of the material predictable, reproducible and precisely defined [9]. These characteristics make synthetic polymers to an interesting raw material for scaffold fabrication [8-10].

In particular, poly-glycolic acid (PGA), poly-lactic acid (PLA), poly-hydroxy alkanoate (PHA), poly -caprolactone (PCL) and their deriving copolymers have generated substantial interest as scaffold materials for the in vitro TE of bone, cartilage, as well as cardiovascular tissues [11-14]. PGA is most commonly used because it degrades at predictable time point and into (generally) biocompatible components [8]. Besides, the high porosity of PGA meshes permits a good diffusion, neovascularization and cellular infiltration [15]. Unfortunately, PGA meshes are biodegraded rapidly within few weeks and can therefore not withstand mechanical forces exerted to the materials and guide the shape of the bioengineered construct over longer culturing periods [15, 16]. As a result, hybrid polymers have been designed in order to combine the shape-memory and mechanical stability of slowly degrading polymers with the fast degrading properties of polymers, such as PGA [17]. For instance, combinations of PGA with polymers such as (poly-4-hydroxybutyrate) P4HB or PLA have been widely explored [8, 13, 14, 18, 19]. PLA is synthesized by polymerization of lactic acid and can be eliminated through the citric acid cycle [15]. Due to the chiral nature of PLA, several distinct forms are existing: poly-L-lactide (PLLA), poly-D-lactide (PDLA) and LD racemic (PDLLA), respectively [15]. PLA can easily be processed and its degradation rates and physical/mechanical

characteristics are adjustable over a wide range by using different molecular weights and copolymers [19, 20]. Also ε-caprolactone and copolymers have been studied intensely and are frequently investigated for biomedical applications [15]. Interestingly, PCL degrades very slowly in-vivo via enzymatic degradation and hydrolysis [21]. Unlike PGA and PCL, which are synthetized using chemical methods, P4HB is produced naturally by microorganisms making it more challenging to be synthesized [22]. After implantation into the body, P4HB degrades mainly by bulk hydrolysis producing 4HB, a normal component of the mammalian body [10].

In 1998, Shinoka et al. reported surgical implantation of tissue engineered vascular grafts (TEVGs) in lambs, in which scaffolds were constructed from autologous cells seeded onto PGA grafts [23]. Further studies have been conducted, for instance, by using PGA-poly-L-lactic acid (PLLA) scaffolds for microvessels in mice [24] or scaffolds composed of polyglycolide knitted fiber, and an L-lactide and ε-caprolactone copolymer sponge for TEVGs in a canine inferior vena cava model [25]. The hybrid polymeric scaffold fabricated from either PGA or PLA fiber-based mesh coated with a 50:50 copolymer of L-lactide and ε-caprolactone (PCLA/PGA or PCLA/PLA) are more elastic than the PGA scaffold [26]. This results in an improved compliance match between the vessel and the conduit and ultimately in better surgical handling characteristics [26]. For both heart valve and vascular tissue engineering the use of PGA meshes coated with P4HB, meaning the combination of the thermoplastic characteristic of P4HB and the high porosity of PGA meshes, has been investigated intensively with promising results in vitro and in preclinical studies [27-29]. In 2006, Hoerstrup et al. provided the first evidence of living, functional pulmonary arteries engineered from vascular cells seeded on PGA/P4HB scaffolds in a growing lamb model [13]. In contrast, PCL has been mainly investigated for biomedical applications in bone and cartilage repair, as surgical suture as well as for drug delivery systems, especially those with longer working lifetimes [12, 15]. Lee et al. showed that a PCL/ poly(methyl methacrylate) (PMMA) scaffold was suitable for cell growth in vitro and for new bone formation in vivo [30]. This study suggested that PCL/PMMA blends can be used for biopolymer composite scaffolds in bone tissue engineering [30]. In general, all polyesters are biocompatible and have formed the bases of numerous FDA approved medical devices for clinical use [9, 31].

However, in spite of their frequent use in biomedical research and therapeutic products, there is still a lack of systematic comparative analyses, such as qualitative and quantitative assays of tissue formation and biomechanics between different synthetic polymers. Therefore, the present study aims at a systematic, multimodal comparison of three frequently used polymers (PGA, PLA, PCL) integrated into a co-polymer solution with P4HB for the in vitro engineering of extracellular matrix under static as well as dynamic conditions. These data might allow for a specific selection of a certain polymer starter matrices aiming at specific tissue properties of bioengineered materials in vitro.

2.2 Material and Methods

2.2.1 Isolation and culture of human umbilical cord fibroblasts

Human umbilical cords (n=3) were collected after full-term births with informed consent according to the cantonal ethics commission of Zurich, Switzerland [KEK-ZH-2009-0095] and processed for isolation of venous fibroblasts according to established protocols [32]. Briefly, the umbilical cord vein was isolated surgically and small tissue pieces were cut out using a dissecting scissors. Tissue pieces were placed on a sterile petri dish and were left to adhere to the bottom for 20min. Culture medium containing DMEM high glucose (Sigma-Aldrich, Switzerland), 10% fetal bovine serum (Biowest, USA) and an antibiotic/antimitotic solution (Sigma-Aldrich, Switzerland), was gently added and changed every third day. Tissue pieces were removed after first cellular outgrowth after approximately 1-2 weeks of incubation under humidified incubator conditions at 5% CO_2 at 37 °C. Three different fibroblast cell lines (named B014, B015 and B020) were isolated.

2.2.2 Phenotyping of human fibroblasts

Isolated human cells (n=3) were characterized using immunofluorescence staining for common myofibroblast markers. Therefore, cells were cultured on 3.5 cm^2 cell culture dishes, fixed with 4% paraformaldehyde (Sigma-Aldrich, Switzerland), and incubated over night at 4°C with the following primary antibodies: alpha smooth muscle actin (1A4, Abcam, United Kingdom), Vimentin (Vim 3B4, Abcam, United Kingdom), CD90 (EPR3133, Abcam, United Kingdom), CD31 (JC70A, Dako, USA), and Phalloidin (Life Technologie, Switzerland). The following secondary antibodies were used: anti mouse Alexa Flour 488 (Invitrogen, USA) and anti rabbit Alexa Flour 488 (Invitrogen, USA) and Dapi (Sigma-Aldrich, Switzerland). Cells were analyzed with a DM6000B fluorescence microscope (Leica, Germany). Image processing was performed using the Leica software (Leica, Germany).

2.2.3 Proliferation assay

Cellular proliferation was assessed by determining the number of total cells based on the absorbance of crystal violet when cultured on a 24-well plate for up to 7 days. In brief, every day cells were fixed with methanol (Sigma-Aldrich, Switzerland) for 10 minutes and stained with 0.1% crystal violet (Artechemis, Switzerland) for 5 minutes. The 24-well plates were washed and air-dried. Cells were solubilized with 2% Na-deoxycholat (Sigma-Aldrich, Switzerland) while being heated at 60°C for 10min. The absorbance was analyzed at 550nm using a standard ELISA reader Synergy HT (Bio TEK, USA). To obtain quantitative information a standard curve with serial dilutions was performed.

2.2.4 Surface morphology of biomaterials

Samples were mounted on electron imagining stubs using carbon tape and subsequently sputter coated in 5nm of Pt/Pd (Quorum Technologies, EMS 300TD, USA) to reduce fiber degradation during imaging. A field emitting electron microscope was used to image samples at 15 kV power (Zeiss, FESEM Ultra Plus) for clarity. For each sample, four regions of interest (ROI) measuring 1000 x 800 um were imaged from which five fibers per ROI were measured using Image J software (NIH, v1.48s, line tool) to determine the average fiber diameter of an ROI. For scaffold porosity, an additional four ROIs measuring 3000 x 2250um were imaged. Each porosity ROI image was then automatically thresholded in Image J and porosity was defined as the percent area that was non-fiber (ie empty/porous space).

2.2.5 Tissue Engineering

2.2.5.1 Scaffold fabrication

Patches were fabricated from non-woven polyglycolic acid (PGA) meshes (thickness 1.0 mm; specific gravity 70 mg/cm3; Cellon, Luxembourg) and coated with either 1% poly-4-hydroxybutyrate (P4HB; TEPHA, Inc., USA) or 1% Poly(L-lactide (PLA, Carbion, USA) or 1% polycaprolactone (PCL, Carbion, USA) by dipping into a tetrahydrofuran solution (Sigma-Aldrich, Switzerland). After solvent evaporation and vacuum drying overnight, the scaffolds were placed into a 70% EtOH (Sigma-Aldrich, Switzerland) for 30 min to obtain sterility, followed by two washing cycles with PBS (Sigma-Aldrich, Switzerland). Thereafter, scaffolds were pre-incubated in DMEM culture medium previously described for 12–24 hours to facilitate cell attachment.

2.2.5.2 Cell seeding

Human fibroblasts (n=3) were seeded onto scaffolds using a density of $1.5*10^6$cells/cm^2. Therefore, fibrinogen (Sigma-Aldrich, Switzerland) (10 mg/mL of active protein) and thrombin (Sigma-Aldrich, Switzerland) were prepared, used and titrated to an optimal clotting time of approximately 30sec by adapting the concentration of fibrinogen. The cells were resuspended in a fibrinogen-thrombin co-solution and subsequently seeded onto the sterile scaffolds. After static incubation of seeded constructs in DMEM (10% fetal bovine serum; penicillin/streptomycin and 0.9mM of L-ascorbic acid-2-phosphate (Sigma-Aldrich, Switzerland)) for 7 days, they were kept either under static conditions or placed onto a shaker for additional mechanical stimulation via shear stress. The constructs were harvested after 21 days of culture and processed immediately for subsequent analyses

2.2.6 Qualitative Tissue Analysis

For immunohistochemical analysis of TE patches (n=3 per group), sections with 5µm thickness derived from blocks of formalin-fixed, paraffin-embedded tissue were mounted on glass slides (SuperFrost Plus, Menzel Gläser,Germany), deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) or Masson Trichrome using standard histological techniques. All sections were analyzed using a Mirax Midi BF slide scanner (Zeiss, Germany) and processed using MIRAX viewer (Zeiss, Germany).

2.2.7 Quantitative Tissue Analysis

TE patches (n = 6 per group) were minced, lyophilized, and analyzed using biochemical assays for total deoxyribonucleic acid (DNA) content as an indicator for cell number, hydroxyproline (HYP) content as an indicator for collagen, as well as for glycosaminoglycan (GAG) content. All TE patches were digested in papain (Sigma-Aldrich, Switzerland) solution (300 µg/mL in PBS with 5mM EDTA (Sigma-Aldrich, Switzerland) and 5mM cysteine (Sigma-Aldrich, Switzerland), at 65°C for 16 hours. For measuring the cellularity of the constructs, the DNA amount was quantified according to manufacturer's protocol (Life Technologies, Switzerland, No. P11496). The GAG content was determined using a modified version of the protocol described by [33], and a standard curve prepared from chondroitin sulfate from shark cartilage (Sigma-Aldrich, Switzerland). Hydroxyproline was determined with a modified version of the protocol provided by [34]. Remnants of the hybrid polymers matrices were visualized using polarization microscopy (Zeiss, Germany).

2.2.8 Biomechanics

After 0 (only for the unseeded group) and 21 days of culture, the mechanical properties of the TE constructs (n = 4 per group) were assessed using a uniaxial tensile tester (Instron 5864, Boston, MA, USA) equipped with a 100-N load cell. For comparison, native tissues (cartilage, skin and vein) were harvested from sheep post mortem provided by the local slaughterhouse Zurich/Hinwil, Switzerland (n=4). The crosshead speed was set to correspond to an initial strain rate of 7mm/min and the tester operated at 5bars of air pressure. Patches had a cross-sectional area of 14mm x 4mm x 1 mm (length x width x thickness) and were fixed with hydraulic clamps. Stress–strain curves were obtained and Young's modulus was determined as the slope of the curve at a strain of 10%, as a measure for tissue stiffness.

2.2.9 Statistic

Quantitative data are presented as mean ± standard deviation. For statistical comparison of the proliferation rate of the three different cell lines a one-way ANOVA was performed and p-values < 0.05 were considered statistically significant. Surface morphology measurements were statistically analyzed by non-parametric Mann-Whitney-U-test and p-values < 0.05 (corrected post hoc according to Bonferroni) were considered statistically significant. In addition, quantitative tissue analysis and biomechanics were statistically evaluated by an unpaired students-t-test and p-values $p < 0.05$ (corrected post hoc according to Bonferroni) were considered statistically significant. KS normality test was used to confirm normal distribution of the dataset ($p > 0.05$). All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software Inc., USA).

2.3 Results

2.3.1 Phenotype and proliferation of umbilical cord derived fibroblasts

Immunofluorescence performed on fibroblasts derived from three different patients revealed that all cell types displayed similar immunophenotypic patterns. Isolated human fibroblasts expressed myogenic markers, such as alpha smooth muscle actin and vimentin, and common fibroblast marker CD90 (Figure 1a).

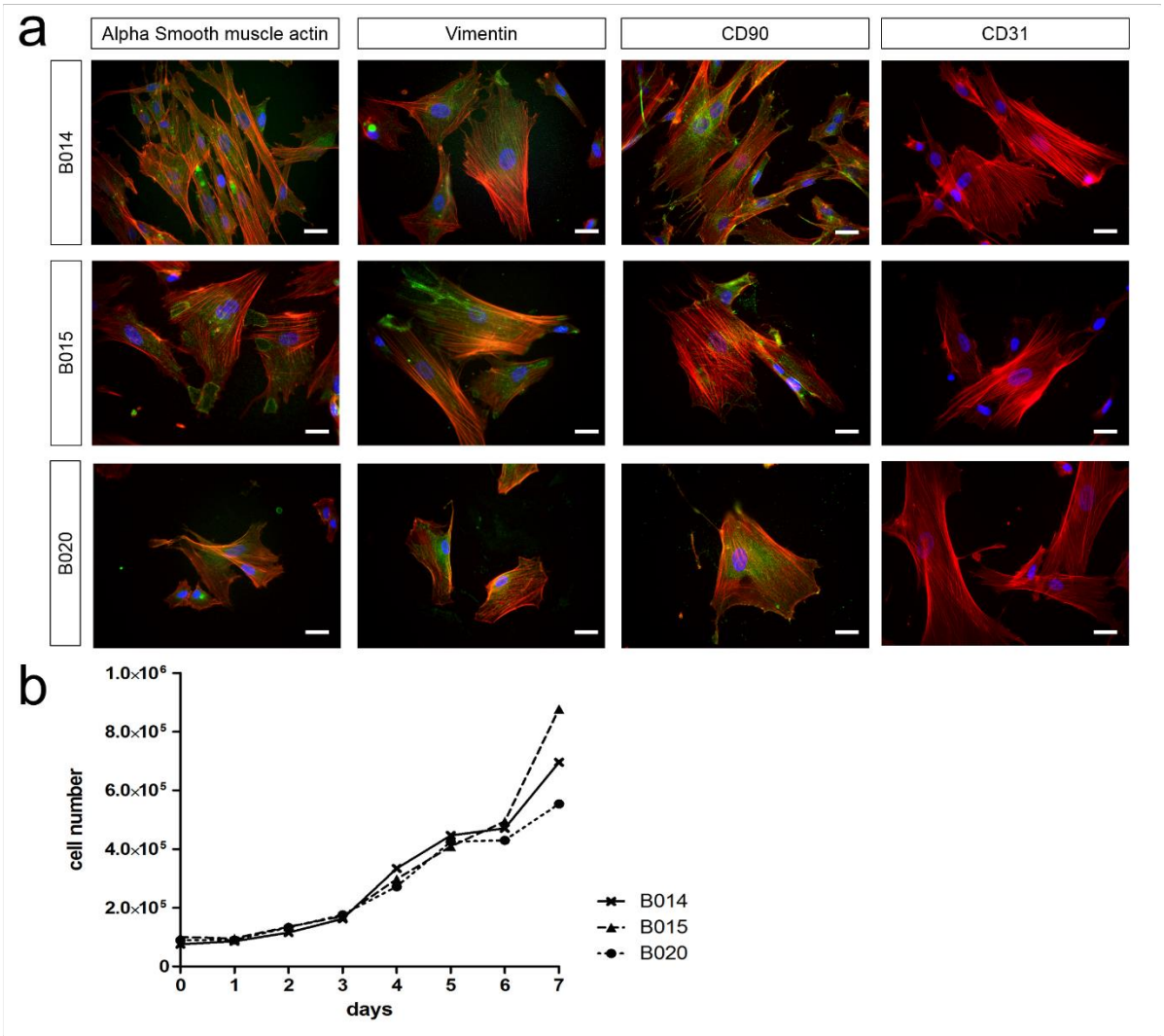

Figure 1: Phenotypic characterization and proliferation of isolated fibroblasts.
a) Immunohistochemical analyses of three different human fibroblast cell lines (B014, B015 and B020) revealed positive expression of alpha smooth muscle actin, Vimentin and CD90 (Phalloidin in red, Dapi in blue and respective antibody in green). All three markers showed a homogenous expression pattern. Fibroblasts were negative for the endothelial cell surface marker CD31. b) Cell number was determined over one week for all three cell lines using the crystal violet-based proliferation assay. No significant differences were detected. (Scale bars: 50 µm)

In contrast, the cells were negative for the endothelial cell surface marker CD31 excluding non-fibroblastic contamination during cell isolation procedures. In the proliferation analysis based on crystal violet staining (Figure 1b), no significant differences in proliferation parameters were detected between the three fibroblast

lines when considering cell numbers over 7 days ($p<0.05$). These findings exclude potential significant inter-individual proliferation differences.

2.3.2 Surface morphology of biomaterials

Microstructural analysis using scanning electron microscopy (SEM) was used to analyze the fiber size and porosity of unseeded polymers before and after coating (Figure 2a-d and f-i) (n=4 per condition).

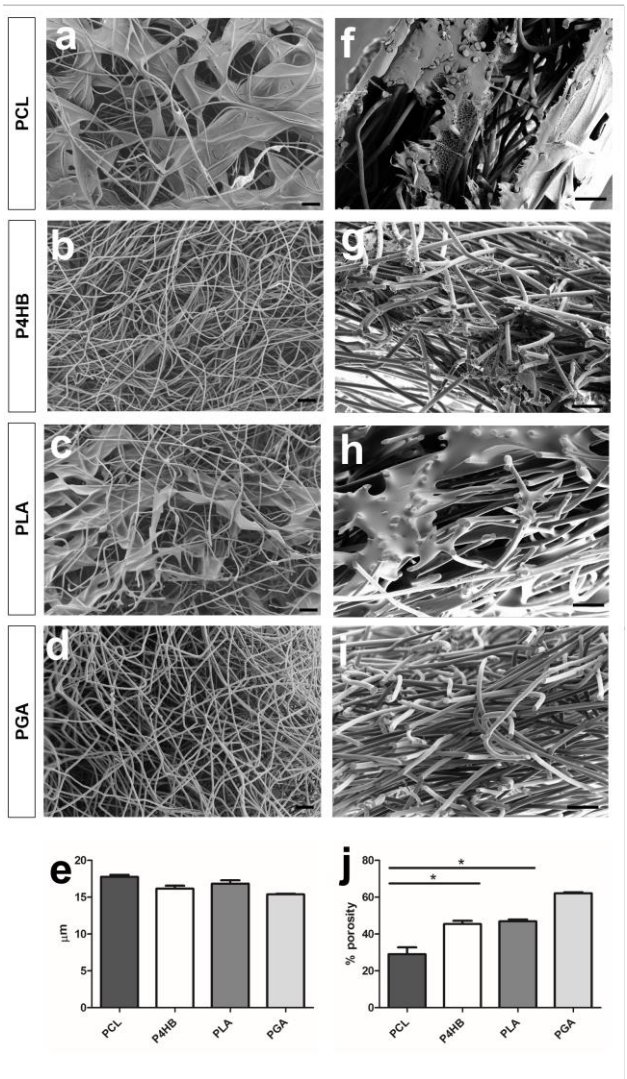

Figure 2: Surface and intersection morphology of biomaterials. Scanning electron micrographs (SEM) show the fiber alignment and size of unseeded biomaterials. a-d) SEM of the surface f-i) SEM of the cross section e) The fiber diameter was consistent among all biomaterials. (n=4) j) PGA-PCL was significantly less porous than PGA-P4HB or PGA-PLA. PGA-P4HB and PGA-PLA showed a similar porosity. (n=4) (Scale bars: 100 µm)

The PGA-PCL matrix (Figure 2a and f) was less porous in comparison to the hybrid polymers PGA-P4HB (Figure 2b and g), PGA-PLA (Figure 2c and h), and PGA (Figure d and i). Quantitative measurements of the porosity confirmed these differences (Figure 2j). PGA-PCL scaffolds were significantly less porous than PGA-P4HB ($p<0.05$) or PGA-PLA ($p<0.05$) scaffolds. PGA-P4HB and PGA-PLA scaffolds showed a similar porosity with no significant difference ($p=0.69$). Uncoated PGA mesh was significant more porous in comparison to PGA-P4HB ($p<0.05$), PGA-PLA ($p<0.05$), and PGA-PCL ($p<0.05$). The fiber diameter was uniform among all biomaterials

with no statistically significant differences between different hybrid polymer groups or uncoated PGA (Figure 2g, $p<0.05$) (Figure 2e). Imaging of the surface (Figure a-d) and cross section (Figure f-i) display the distribution of the coating compared to PGA scaffold only. The different hybrid polymer present a similar distribution of the coating. In general, there was more coating visible on the outer scaffold regions, while in the central part of the scaffold less was detected.

2.3.4 Qualitative Tissue Analyses

Microstructural features of human fibroblast-derived TE patches were analyzed by histological staining procedures (Figure 3) as well as polarization microscopy (Figure 4).

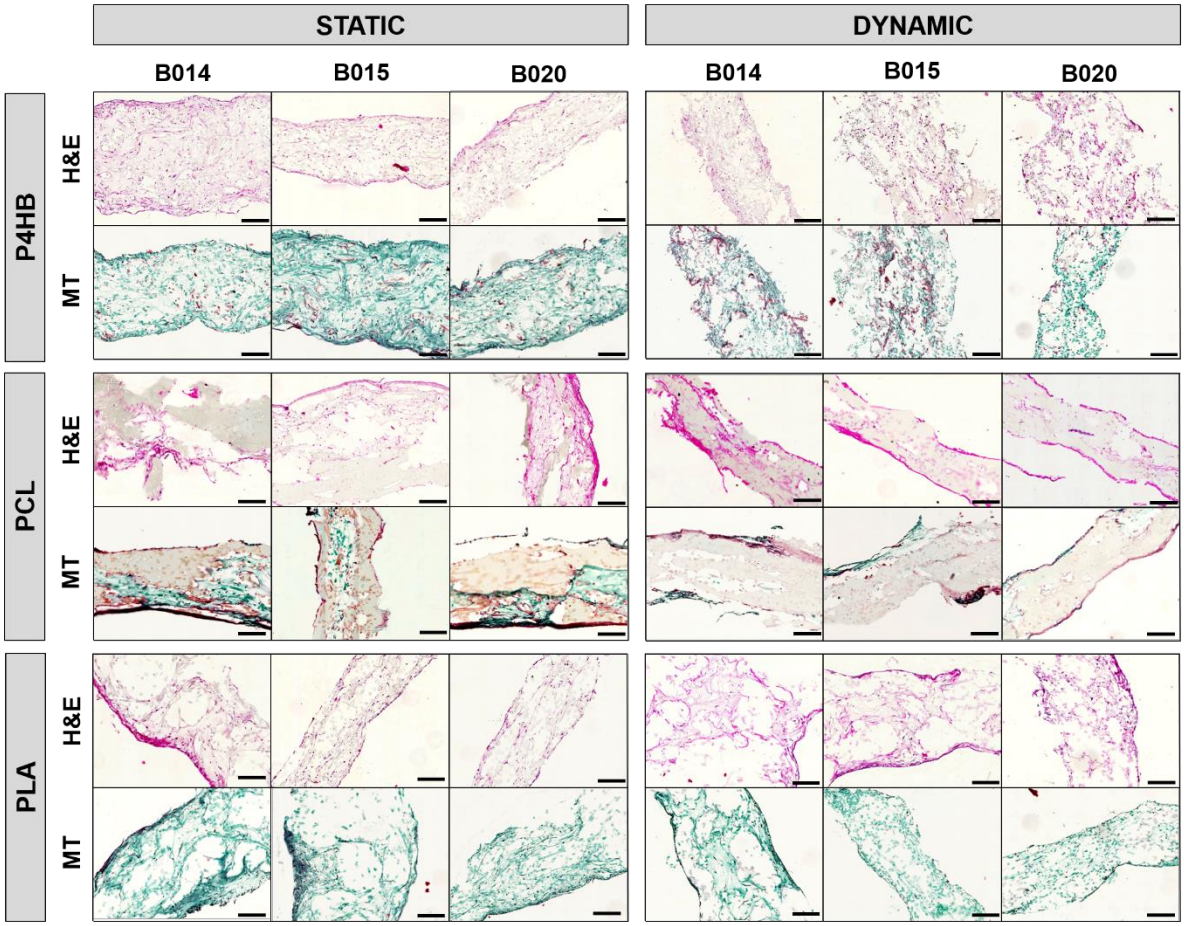

Figure 3: Histology of tissue-engineered patches under static and dynamic conditions. Hematoxylin-eosin (H&E) staining of statically cultured tissue-engineered patches (left column) revealed cellular ingrowth with tissue formation, and some collagen fibers could be detected with Masson Trichrome staining (MT). In contrast, patches cultured under dynamic conditions (right column) demonstrated more cellular tissue formation and production of extracellular matrix elements. All three cell lines displayed similar tissue formation for respective conditions/coatings. PGA-PCL patches showed for both culture conditions less tissue formation and high quantities of scaffold remnants in comparison to PGA-P4HB or PGA-PLA. (Scale bars: 200 µm)

H&E staining demonstrated formation of extracellular matrix (ECM) in vitro with high cellularity and layered tissue on the outer scaffold regions, while in the central part of the scaffold low cellularity and no significant formation of ECM were present. In order to investigate the deposition of collagen fibers Masson Trichom

staining was used. In general, TE patches under dynamic conditions (Figure 3, right column) revealed more tissue formation and ECM deposition compared to static conditions.

Importantly, all three cell lines exhibited comparable tissue formation for respective hybrid polymer conditions excluding a potential bias due to inter-individual differences. PGA-PCL-based patches showed for both culture conditions (dynamic and static) less tissue formation and high quantities of scaffold remnants in comparison to the PGA-P4HB or PGA-PLA. In order to confirm these findings polarization microscopy was performed to further visualize different polymer components (Figure 4).

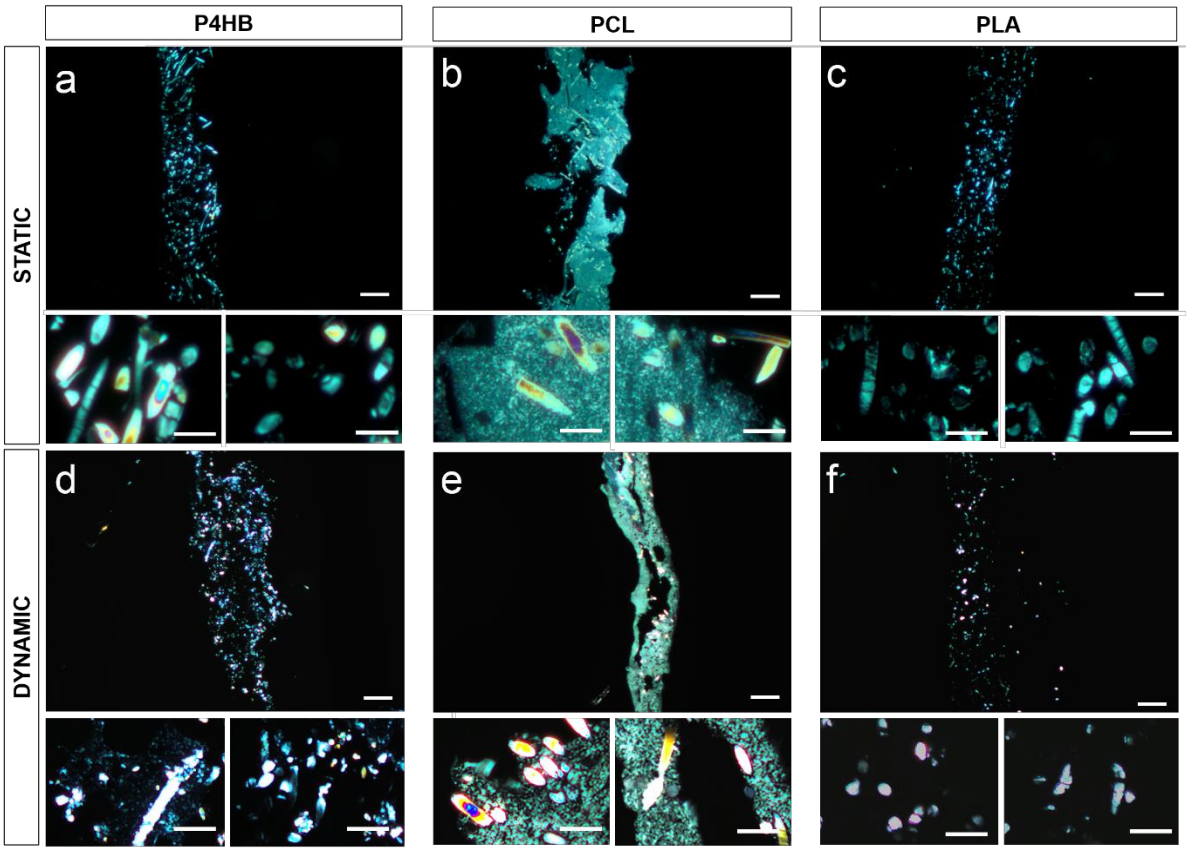

Figure 4: Polarization microscopy of tissue-engineered patches under static and dynamic conditions. Polarization microscopy revealed the presence of the initial scaffold matrix in particular in the central part of the constructs. PGA fibers are shown as elongated ellipses (high magnification). Webbings between PGA fibers represent coating remnants (low magnification). However, in the dynamic fibroblast-based constructs the biomaterial remnants were more degraded (d-f) compared with static cultures (a-c). PGA-PCL (b, e) starter matrix showed a very strong preservation of the co-polymer when compared to PGA-P4HB (a, d) or PGA-PLA (c, f) scaffolds (scale bars: 400µm low magnification, 100 µm high magnification).

The starter matrices showed no major remodeling in the central part of the constructs in static as well as dynamic conditions given the lack of tissue formation in this area of the constructs. PGA fibers were visible as elongated ellipses (Figure 4, high magnification), whereas the webbings between PGA fibers represented remnants of the coating (Figure 4, low magnification). In dynamic conditions the biomaterial was more degraded (Figure 4d-f) when compared to the static cultures (Figure 4a-c). In particular, the PGA-PCL starter

matrix (Figure 4b, e) showed strong preservation of the polymer components compared to PGA-P4HB (Figure 4a, d) or PGA-PLA (Figure 4c, f).

2.3.5 Quantitative Tissue Analysis

The composition of the ECM of the human fibroblast-derived TE constructs was biochemically analyzed using assays for HYP, GAG, and the cell number (DNA) (Figure 5).

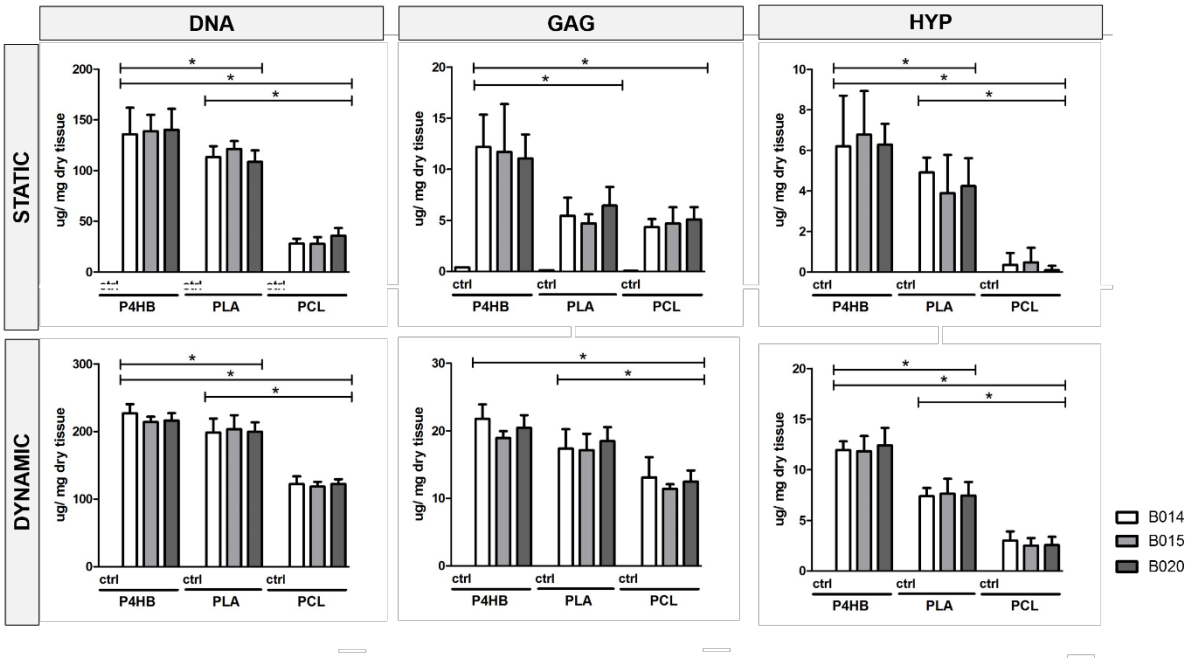

Figure 5: Extracellular matrix analysis of tissue-engineered patches under static and dynamic conditions. Extracellular matrix analysis shows the amount of hydroxyproline (HYP), glycosaminoglycans (GAG), and the cell number (deoxyribonucleic acid, DNA) of tissue-engineered patches, which were statically or dynamically cultured (all values relative to control samples (ctrl) indicating biomaterials only). Generally, all three cell lines displayed similar amounts of HYP, GAG and DNA for respective conditions/coatings, no significant differences were detected. Overall PGA-P4HB (n=6) showed a higher expression of DNA, GAG, and HYP in comparison to PGA-PLA (n=6) or PGA-PCL (n=6).

The expression level of DNA, GAG, and HYP is for all 3 coatings higher under dynamic conditions than under static conditions. Analyses were performed after three weeks of static or dynamic culture. In general, no significant differences were detected between the three different cell lines relating to expression of HYP, GAG and DNA for respective coatings under static or dynamic conditions, indicating no inter-individual differences. In total, six TE patches per cell line and per coating condition were analyzed. PGA-P4HB showed a significantly higher expression of DNA, GAG, and HYP in comparison to PGA-PLA ($p<0.05$) or PGA-PCL ($p<0.05$) under static conditions suggesting a more rapid formation of ECM in vitro. Moreover, PGA-PLA exhibited a significantly higher expression of DNA and HYP than PGA-PCL ($p<0.05$) under static conditions. Dynamically cultured PGA-PLA ($p<0.05$) and PGA-P4HB ($p<0.05$) patches expressed a significantly higher amount of DNA, GAG and HYP than PGA-PCL patches. PGA-P4HB ($p<0.05$) also displayed a significantly higher

DNA and HYP expression than PGA-PLA. In general, the expression levels of DNA, GAG and HYP were higher for all coatings under dynamic conditions compared to statically cultured patches.

2.3.6 Biomechanics

The material properties of the different TE patches used in this study were determined via uniaxial tensile tests in comparison to native ovine tissue, such as cartilage, skin and vein (Figure 6). Stress–strain curves were obtained and Young's modulus was determined as the slope of the curve at a strain of 10%, as a measure for tissue stiffness. Contribution of tissue formation to the mechanical properties was observed in all TE patches, given a higher elasticity with culture time in comparison to unseeded control. Due to the rapid loss of mechanical integrity of the scaffold, biomechanical tests on the TE patches under dynamic conditions over time could not be performed.

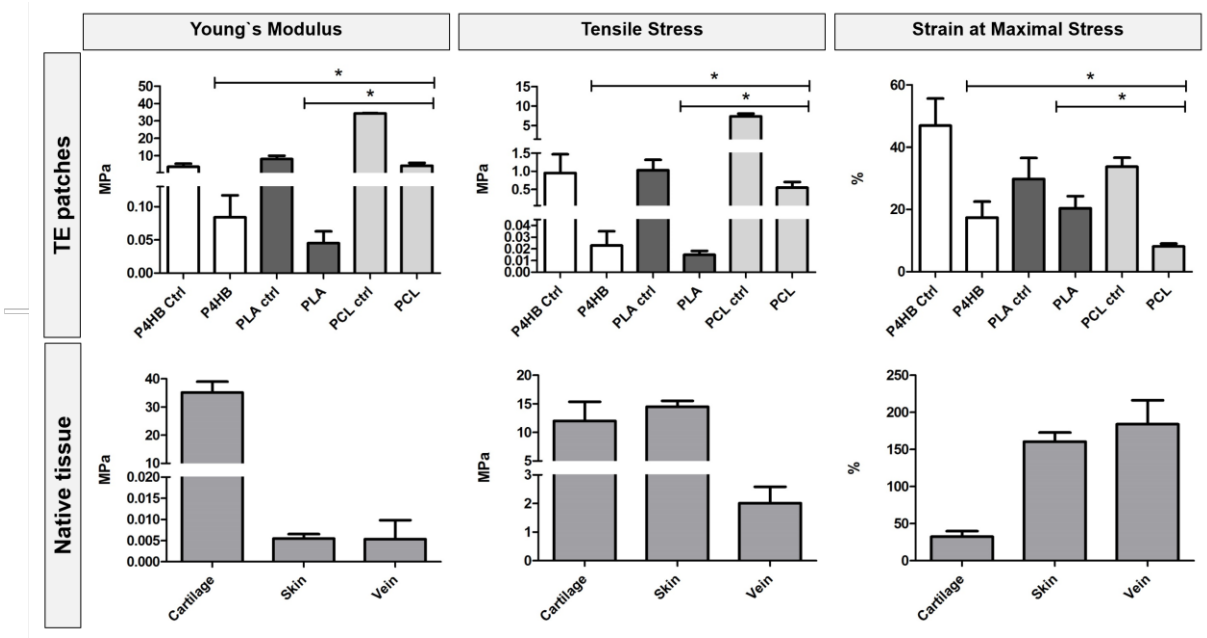

Figure 6: Mechanical properties of tissue-engineered patches and native tissue. Stress–strain curves were obtained and Young's modulus was determined as the slope of the curve at a strain of 10%, as a measure for tissue stiffness. Control (ctrl) means biomaterials only. Generally, PGA-PCL (n=12, 4 patches per group) showed a significant higher Young`s modulus, tensile strength and lower strain at maximal stress compared to PGA-PLA (n=12) or PGA-P4HB (n=12). As a comparison cartilage, skin and vein samples (n=4) were also tested.

Overall, PGA-PLA and PGA-P4HB TE patches show similar biomechanical properties. Both conditions display tensile stress of about 0.02MPa and strain at maximal stress of about 20%. Tensile moduli of both 0.04MPa and 0.08MPa were obtained, which were not significantly different ($p=0.1479$). In case of PGA-PCL, tensile stress was 0.54MPa, tensile strain was 8%, and having a Young`s modulus of 4MPa respectively. Generally, PGA-PCL showed a significantly higher Young`s modulus, tensile strength and lower strain at maximal stress compared to PGA-PLA ($p<0.05$) or PGA-P4HB ($p<0.05$). For a better interpretation of the obtained results, also native ovine tissue were analyzed biomechanically. For cartilage, Young's modulus was about 35MPa, tensile strength was 12MPa, and strain at maximal stress was 32.5%. In addition, skin had a Young`s modulus

of 0.005MPa, tensile stress of 14.5MPa and tensile strain of 160%. Similar results were found for the vein with a Young`s of 0.0054MPa, a tensile stress of 2MPa and tensile strain of 185%.

2.4 Discussion

The tissue formation produced by implanted cells is highly influenced by the scaffold onto which they are seeded. Different types of biodegradable polymers as a scaffold have been investigated for tissue regeneration, such as PGA, PLA, P4HB and PCL [8, 10, 20]. Therefore, the present study aimed at a systematic, multimodal comparison of three frequently used polymers for the in vitro engineering of extracellular matrix under static as well as dynamic conditions.

So far, most biomaterials do not completely mimic physiological microenvironment and can therefore not enable native like cellular interaction and behavior [15]. Blending two polymers has gathered growing interest for constructing native-like tissues [15]. Thus, patches were cut from PGA meshes and coated with same concentrations of either P4HB, PLA, or PCL. In addition, to mimic the native tissue and serving as an artificial environment, the structure and surface morphology of the scaffolds have to meet general requirements: (I) pores to ensure cell growth and nutrients/ metabolic waste transport; (II) three-dimensional architecture; (III) suitable mechanical properties depending on the native tissue to be replaced; (IV) suitable surface chemistry; (V) biodegradation and bioresorbability [35]. Moreover, pore size affects cell binding, migration depth of cellular in-growth, cell morphology, and phenotypic expression [36].

For this reason, we performed SEM to analyze the ultra-structure of the unseeded scaffold. SEM confirmed the uniform fiber diameters of the coated PGA matrix. In contrast, PGA-PCL scaffolds were significantly less porous than PGA-P4HB or PGA-PLA scaffolds. PGA-P4HB and PGA-PLA scaffolds showed a similar porosity. These findings already suggested a potential improved cell infiltration and consequent tissue formation for PGA-P4HB and PGA-PLA in comparison to PGA-PCL. For example, porosity of the scaffold plays an important role in bone and cartilage regeneration [37, 38]. Interestingly, lower porosity enhances osteogenesis by suppressing cell proliferation and forcing cell aggregation in vitro [38]. In contrast, higher porosity and larger pore size lead to greater bone ingrowths to the scaffold but diminish the mechanical properties in vivo [38]. However, one has to take into account that for comparative issues the concentrations of P4HB, PLA, and PCL were consistent and could be further adapted for specific requirements.

Hence, microstructural features of human fibroblast-derived TE patches were analyzed by histological stainings as well as polarization microscopy. In general, tissue engineered patches under dynamic conditions represented a more extensive tissue formation and ECM deposition. Wang et al. also showed an enhanced cell proliferation under dynamic three-dimensional (3D) culture compared with conventional static two-dimensional (2D) and 3D cell culture conditions [39]. In addition, high-throughput microarray analysis showed that gene expressions of dynamic 3D system displayed significant differences in cell proliferation and differentiation compared with static-2D conditions [39]. Nonetheless, PGA-PCL based patches displayed less tissue formation and higher quantities of scaffold remnants in comparison to the PGA-P4HB or PGA-PLA under static as well as under dynamic conditions.

In general, dynamic conditions enhanced the degradation of biomaterial in comparison to static conditions. In particular, less PGA fibers were detectable after 3 weeks of culture in comparison to the webbings between the PGA fibers, especially PGA-PCL showed particularly strong preservation of the polymer components. Different studies have proven that PGA degrades faster than P4HB, PLA and PCL [40, 41]. In addition, production conditions of synthetic polymers can be tightly controlled, hence making mechanical and physical properties well defined and predictable. For instance, the time to complete degradation and resorption of P4HB varies with size and processing, i.e., the orientation of the polymer fibers. In general, complete resorption of P4HB occurs between 12 and 18 months in vivo [40]. The characteristics of PLA are highly affected by the stereo-isomeric L/D ratio of lactate units [8]. Generally, an increased stereo-isomeric ratio decreases the crystallinity, whereby the degradation is enhanced. For example, degradation of PLA is faster than PDLA due to the lower crystallinity of PLA [8]. PLA and PCL degradation takes longer than 24 months in vivo [41]. In contrast, PGA is degraded within 4-6 months in vivo [15, 16]. The composition of the ECM of human fibroblast-derived TE constructs was analyzed and PGA-P4HB showed a significantly higher expression of DNA, GAG, and HYP in comparison to PGA-PLA or PGA-PCL under static conditions suggesting a more rapid formation of ECM in vitro. The interactions between cells and ECM are essential in cell attraction, cell differentiation and wound healing [42]. Dynamically cultured PGA-PLA and PGA-P4HB patches expressed a significantly higher amount of DNA, GAG and HYP than PGA-PCL patches. The general expression levels of DNA, GAG and HYP were higher for all coatings under dynamic conditions compared to statically cultured patches. These findings confirm and underline the findings in histology. It is proven that mechanobiological interactions between cells and scaffolds can crucially influence cell behavior [43-45]. For example, the mechanobiologic regulation of cartilage matrix biosynthesis was successfully exploited by the application of cyclic compression to constructs formed by encapsulating primary bovine chondrocytes in agarose hydrogels [45, 46]. By 28 days, determined improvements in the compressive stiffness were observed in groups that had been exposed to mechanical loading compared with free swelling controls [46]. Even though relations between these forces and cell responses are exhibited, elucidation of the mechanotransduction pathways is far from revealed. The transduction of external forces to signaling pathways involves intracellular forces and associated molecular deformations, such as gap junctions on the cell membrane, the cytoskeleton, and DNA [44, 45].

Importantly, a balance between the rate of scaffold degradation and tissue formation is crucial for maintaining mechanical integrity of the replaced tissues [47]. The biodegradable scaffold should have sufficient mechanical properties (such as strength and stiffness) approximating those of the host tissue until the biodegradable scaffold matrix is substituted by the new tissue [47]. The material properties of different TE patches used in this study were determined via uniaxial tensile tests. In addition, we compared these values with native ovine cartilage, skin and vein tissue. Contribution of tissue formation to the mechanical properties was observed in all TE patches, as samples became less stiff with culture time in comparison to unseeded controls. Overall, PGA-PLA and PGA-P4HB TE patches show similar biomechanical properties.

Generally, PGA-PCL showed a significant higher Young`s modulus, tensile strength and lower strain at maximal stress compared to PGA-PLA or PGA-P4HB. Meaning that on one hand PGA-PCL is stiffer and on the other hand is more robust than PGA-P4HB and PGA-PLA. Notably, in contrast to PGA-PCL, PGA-P4HB and PGA-PLA are more ductile and flexible. The values are still not in the range of native tissue but this may rely on the polymer remnants and the incomplete tissue formation in vitro. However, as with many polymers, the mechanical properties of the material depend not only on the basal material, but also on its processing history [40].

Conclusion

Biodegradable synthetic polymers are an interesting raw material for scaffold fabrication and have been intensively investigated for TE. As the scaffold plays a crucial role in the successful design of TE constructs, the choice of material directly influences the outcome. Our study may allow for a specific selection of a certain polymer starter matrices aiming at specific tissue properties of bioengineered materials in vitro. In general, we showed that PGA-P4HB coating display better tissue formation and a significant higher expression level of DNA, GAG and HYP in comparison to PGA-PLA and PGA-PCL. However, PGA-PCL is under biomechanical conditions more robust than PGA-P4HB and PGA-PLA.

Future studies are required to evaluate, which combination under which conditions allows for the best tissue formation and native-like biomechanical properties.

Acknowledgments

The authors would like to thank Nicole Gampp, Ursula Steckholzer, Nicole Gross and Ulrich Bleul for their technical help and assistance. Furthermore, we would like to thank Kirill Feldmann for the biomechanical advice and assistance. The research leading to these results has received funding from the Swiss National Science Foundation (Project 310030_143992), Forschungskredit Candoc of the University of Zurich, Forschungskredit Postdoc of the University of Zurich, the Foundation for Research in Science and the Humanities at the University of Zurich and the Alfred and Anneliese Sutter-Stöttner-Foundation.

2.5 References

1. Langer, R. and J.P. Vacanti, *Tissue engineering.* Science, 1993. **260**(5110): p. 920-6.
2. Yuan, P., et al., *Design, development and clinical validation of computer-aided surgical simulation system for streamlined orthognathic surgical planning.* Int J Comput Assist Radiol Surg, 2017.
3. Li, Q., L. Ma, and C.Y. Gao, *Biomaterials for in situ tissue regeneration: development and perspectives.* Journal of Materials Chemistry B, 2015. **3**(46): p. 8921-8938.
4. Hu, B., C. Ye, and C.Y. Gao, *Synthesis and characterization of biodegradable polyurethanes with unsaturated carbon bonds based on poly(propylene fumarate).* Journal of Applied Polymer Science, 2015. **132**(24).
5. Bi, X.P., et al., *A functional polyester carrying free hydroxyl groups promotes the mineralization of osteoblast and human mesenchymal stem cell extracellular matrix.* Acta Biomaterialia, 2014. **10**(6): p. 2814-2823.
6. Cai, W.Y., et al., *Carboxyl-ebselen-based layer-by-layer films as potential antithrombotic and antimicrobial coatings.* Biomaterials, 2011. **32**(31): p. 7774-7784.
7. Philipp, A., et al., *Functional modification of amide-crosslinked oligoethylenimine for improved siRNA delivery.* Reactive & Functional Polymers, 2011. **71**(3): p. 288-293.
8. Dijkman, P.E., et al., *Polymeric Starter Matrices for Cardiovascular Tissue Engineering.* Encyclopedia of Biomedical Polymers and Polymeric Biomaterials, 2015(CRC Press 2015): p. 1–25.
9. Sensharma, P., et al., *Biomaterials and cells for neural tissue engineering: Current choices.* Mater Sci Eng C Mater Biol Appl, 2017. **77**: p. 1302-1315.
10. Chen, G.Q. and Q. Wu, *The application of polyhydroxyalkanoates as tissue engineering materials.* Biomaterials, 2005. **26**(33): p. 6565-78.
11. Nooeaid, P., et al., *Osteochondral tissue engineering: scaffolds, stem cells and applications.* J Cell Mol Med, 2012. **16**(10): p. 2247-70.
12. Rentsch, B., et al., *Embroidered and surface coated polycaprolactone-co-lactide scaffolds: a potential graft for bone tissue engineering.* Biomatter, 2012. **2**(3): p. 158-65.
13. Mol, A., et al., *Autologous human tissue-engineered heart valves: prospects for systemic application.* Circulation, 2006. **114**(1 Suppl): p. I152-8.
14. Weber, B., et al., *Injectable living marrow stromal cell-based autologous tissue engineered heart valves: first experiences with a one-step intervention in primates.* Eur Heart J, 2011. **32**(22): p. 2830-40.
15. Asti, A. and L. Gioglio, *Natural and synthetic biodegradable polymers: different scaffolds for cell expansion and tissue formation.* International Journal of Artificial Organs, 2014. **37**(3): p. 187-205.
16. Dunn, A.S., P.G. Campbell, and K.G. Marra, *The influence of polymer blend composition on the degradation of polymer/hydroxyapatite biomaterials.* Journal of Materials Science-Materials in Medicine, 2001. **12**(8): p. 673-677.
17. Sugiura, T., et al., *Tropoelastin Inhibits Intimal Hyperplasia of Mouse Bioresorbable Arterial Vascular Grafts.* Circulation, 2016. **134**.
18. Wu, Q., Y. Wang, and G.Q. Chen, *Medical application of microbial biopolyesters polyhydroxyalkanoates.* Artif Cells Blood Substit Immobil Biotechnol, 2009. **37**(1): p. 1-12.
19. Saini, P., M. Arora, and M.N. Kumar, *Poly(lactic acid) blends in biomedical applications.* Adv Drug Deliv Rev, 2016. **107**: p. 47-59.
20. Engelberg, I. and J. Kohn, *Physico-mechanical properties of degradable polymers used in medical applications: a comparative study.* Biomaterials, 1991. **12**(3): p. 292-304.
21. Couet, F., N. Rajan, and D. Mantovani, *Macromolecular biomaterials for scaffold-based vascular tissue engineering.* Macromol Biosci, 2007. **7**(5): p. 701-18.
22. Li, H., R. Du, and J. Chang, *Fabrication, characterization, and in vitro degradation of composite scaffolds based on PHBV and bioactive glass.* J Biomater Appl, 2005. **20**(2): p. 137-55.
23. Shinoka, T., et al., *Creation of viable pulmonary artery autografts through tissue engineering.* J Thorac Cardiovasc Surg, 1998. **115**(3): p. 536-45; discussion 545-6.

24. Wu, X., et al., *Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells.* Am J Physiol Heart Circ Physiol, 2004. **287**(2): p. H480-7.
25. Matsumura, G., et al., *Long-term results of cell-free biodegradable scaffolds for in situ tissue-engineering vasculature: in a canine inferior vena cava model.* PLoS One, 2012. **7**(4): p. e35760.
26. Watanabe, M., et al., *Tissue-engineered vascular autograft: inferior vena cava replacement in a dog model.* Tissue Eng, 2001. **7**(4): p. 429-39.
27. Schmidt, D., U.A. Stock, and S.P. Hoerstrup, *Tissue engineering of heart valves using decellularized xenogeneic or polymeric starter matrices.* Philos Trans R Soc Lond B Biol Sci, 2007. **362**(1484): p. 1505-12.
28. Hoerstrup, S.P., et al., *Functional living trileaflet heart valves grown in vitro.* Circulation, 2000. **102**(19 Suppl 3): p. III44-9.
29. Weber, B., et al., *Off-the-shelf human decellularized tissue-engineered heart valves in a non-human primate model.* Biomaterials, 2013. **34**(30): p. 7269-80.
30. Son, S.R., et al., *In vitro and in vivo evaluation of electrospun PCL/PMMA fibrous scaffolds for bone regeneration.* Science and Technology of Advanced Materials, 2013. **14**(1).
31. Thomas, L.V., V. Lekshmi, and P.D. Nair, *Tissue engineered vascular grafts--preclinical aspects.* Int J Cardiol, 2013. **167**(4): p. 1091-100.
32. Hoerstrup, S.P., et al., *Living, autologous pulmonary artery conduits tissue engineered from human umbilical cord cells.* Ann Thorac Surg, 2002. **74**(1): p. 46-52; discussion 52.
33. Farndale, R.W., D.J. Buttle, and A.J. Barrett, *Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue.* Biochim Biophys Acta, 1986. **883**(2): p. 173-7.
34. Huszar, G., J. Maiocco, and F. Naftolin, *Monitoring of collagen and collagen fragments in chromatography of protein mixtures.* Anal Biochem, 1980. **105**(2): p. 424-9.
35. Olah, L. and L. Borbas, *Properties of calcium carbonate-containing composite scaffolds.* Acta Bioeng Biomech, 2008. **10**(1): p. 61-6.
36. O'Brien, F.J., et al., *The effect of pore size on cell adhesion in collagen-GAG scaffolds.* Biomaterials, 2005. **26**(4): p. 433-41.
37. Frenkel, S.R., et al., *Regeneration of articular cartilage - Evaluation of osteochondral defect repair in the rabbit using multiphasic implants.* Osteoarthritis and Cartilage, 2005. **13**(9): p. 798-807.
38. Karageorgiou, V. and D. Kaplan, *Porosity of 3D biomaterial scaffolds and osteogenesis.* Biomaterials, 2005. **26**(27): p. 5474-5491.
39. Tang, Y., et al., *The combination of three-dimensional and rotary cell culture system promotes the proliferation and maintains the differentiation potential of rat BMSCs.* Sci Rep, 2017. **7**(1): p. 192.
40. Williams, S.F., S. Rizk, and D.P. Martin, *Poly-4-hydroxybutyrate (P4HB): a new generation of resorbable medical devices for tissue repair and regeneration.* Biomed Tech (Berl), 2013. **58**(5): p. 439-52.
41. Gunatillake, P.A. and R. Adhikari, *Biodegradable synthetic polymers for tissue engineering.* Eur Cell Mater, 2003. **5**: p. 1-16; discussion 16.
42. Phadke, A., C.W. Chang, and S. Varghese, *Functional Biomaterials for Controlling Stem Cell Differentiation.* Biomaterials as Stem Cell Niche, 2010. **2**: p. 19-44.
43. Janmey, P.A. and C.A. McCulloch, *Cell mechanics: integrating cell responses to mechanical stimuli.* Annu Rev Biomed Eng, 2007. **9**: p. 1-34.
44. Guilak, F., et al., *Biomechanics and mechanobiology in functional tissue engineering.* J Biomech, 2014. **47**(9): p. 1933-40.
45. Butler, D.L., et al., *The impact of biomechanics in tissue engineering and regenerative medicine.* Tissue Eng Part B Rev, 2009. **15**(4): p. 477-84.
46. Mauck, R.L., et al., *Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels.* J Biomech Eng, 2000. **122**(3): p. 252-60.
47. Chung, H.J. and T.G. Park, *Surface engineered and drug releasing pre-fabricated scaffolds for tissue engineering.* Adv Drug Deliv Rev, 2007. **59**(4-5): p. 249-62.

Chapter 3

Endothelialized bioengineered vascular grafts based on peripheral blood mononuclear cell-derived induced pluripotent stem cells

Melanie Generali[1], Elisa A. Casanova[2], Debora Kehl[1], Peter K. Bode[3], Simon P. Hoerstrup[1], Paolo Cinelli[2,4,5*] and Benedikt Weber[1,4,6*]

* These senior authors contributed equally to this study

[1]Institute for Regenerative Medicine (IREM), Center for Therapy Development and Good Manufacturing Practice, University of Zurich, Zurich, Switzerland
[2]Division of Trauma Surgery, Center for Clinical Research, University Hospital Zurich, University of Zurich, Zurich, Switzerland
[3]Institute of Surgical Pathology, University Hospital Zurich, Zurich, Switzerland
[4]Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich, Zurich, Switzerland
[5]Institute of Laboratory Animal Science, University of Zurich, Zurich, Switzerland
[6]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

Submitted

3.1 Introduction

Cardiovascular disease remains to be the leading cause of morbidity and mortality, accounting for 17.3 million deaths per year worldwide [1]. Replacement of affected vascular tissues has been widely used to treat cardiovascular disease such as coronary heart disease, aortic aneurysm and peripheral vascular disease [2]. However, successful treatment of cardiovascular disease is often limited by the lack of suitable autologous replacement tissue. So far, expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (PET) and polyurethane (PU) have been used successfully to produce synthetic vascular grafts with a diameter greater than 6 mm [3, 4]. Grafts smaller than 6 mm in diameter lead to many complications including acute thrombosis and stenosis caused by the lack of functional endothelium coverage [5]. Several approaches focusing on the prevention of thrombogenicity of these synthetic materials have been undertaken; in particular, coating of the luminal surface with heparin and other anticoagulant materials has been attempted with limited success [6, 7]. In addition, these grafts do not show growth-adaptive behavior, which would be of particular importance in children that require multiple reoperations due to the lack of growth potential [8]. Therefore, the current synthetic vascular replacements are suboptimal and tissue engineering (TE) is proposed as a solution by replacing tissues or organs with functional autologous replacement constructs. Tissue-engineered vascular grafts (TEVGs) represent living vascular replacement constructs, where vascular cells are seeded onto a three-dimensional biodegradable scaffold and then stimulated via conditioning in a bioreactor to promote tissue formation *in vitro*. However, mature vascular cells are associated with limited access, expansion and differentiation potential [9]. Therefore, different cell sources have been tested in recent years also with a focus on their use for vascular tissue engineering [10, 11]. In general, the vascular cell source seems to be a central problem for cell-based vascular therapies as isolated vascular cells from donor tissues are insufficient in quantity and show limited proliferation, extra cellular matrix (ECM) formation capacity, and cellular functionality following the extensive *in vitro* expansion process [12]. SMCs can be differentiated from alternative cell sources such as bone-marrow mesenchymal stem cells [13, 14], endothelial progenitor cells [15], or adipose-derived stem cells [16]. Nonetheless, the proliferation and differentiation capabilities of adult stem cells considerably decline with aging donors [17]. An interesting alternative is the use of human induced pluripotent stem cells (hiPSCs) which show an equivalent proliferation capacity, differentiation potential and gene expression similar to embryonic stem cells (ESCs). The main benefits of iPSCs is that they are able to differentiate into any cell type of the human body and thus may represent an ideal cell source for tissue engineering largely independent of the age of the respective donor. hiPSCs can be generated from potentially any terminally differentiated cell of the human body [18]. Generally, the possibility to reprogram somatic cells from a patient and generate ‘customized’ tissues from only few cells taken has created substantial hope in the field of regenerative medicine. The created autologous organs, organoids or tissues can then be transplanted back into the respective patient.

Furthermore, hiPSCs provide an unlimited source of proliferating cells and overcome the limitations of confined donor cell availability as well as limited proliferation capacity.

Since the first iPSCs were generated from skin fibroblasts, many other sources of adult somatic cells have been tested for their suitability for reprogramming [18]. The use of peripheral blood mononuclear cells (PBMCs) isolated from peripheral blood as a source for the derivation of hiPSCs has many benefits. Firstly, blood collection is a minimal invasive procedure compared to a skin biopsy. Second, blood allows a minimally invasive large-scale isolation of sterile, viable, autologous, cellular material. A further advantage is that PBMCs can be reprogrammed immediately after extraction [19] whereas dermal fibroblasts need to be first expanded *in vitro.*

The present study aims at the generation of TEVGs based on SMCs and ECs differentiated from PBMC-derived hiPSC combined with a fully biodegradable polymer matrix based on PGA and P4HB. These results pave the way for developing autologous vascular replacements, as well as a basis for modelling human disease *in vitro* using blood as a the only, easily accessible cell source.

3.2 Material and Methods

3.2.1 Isolation and reprogramming of PBMC

Human peripheral blood was collected with written informed consent according to the permission from the cantonal ethics commission of Zurich, Switzerland [KEK-ZH-2014-0430]. PBMCs were isolated according to standard protocols using Ficoll-Paque method. The reprogramming of PBMCs into hiPSCs was induced according to previously reported protocol [34]. Shortly, PBMCs reprogramming was achieved using viral vectors, which lead to the simultaneous expression of the specific transcription factors Oct4, Sox2, c-Myc and Klf4 (STEMCCA lentiviral vector, Boston University School of Medicine, Boston, USA). The infected cells were then seeded on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells and cultured in human iPSCs medium (DMEM/F12 (Invitrogen, Switzerland), 20% Knockout serum replacement (Invitrogen, Switzerland), 100mM nonessential amino acids (Sigma, Switzerland), 50mM 2-mercaptoethanol (Life Technologies, Switzerland), 1mM L-glutamine (Sigma, Switzerland), 50 U/ml penicillin/streptomycin (Sigma, Switzerland), 10ng/ml basic fibroblast growth factor (bFGF) (Life Technologies, Switzerland)). The reprogramming process took about 15-30 days and finally, embryonic stem like colonies were picked and expanded.

3.2.2 Characterization of PBMC derived iPSCs

Four different hiPSC lines were characterized using a standard series of quality controls as follows:

- **Quantitative Real-Time PCR** (qRT-PCR): Total RNA was extracted using RNeasy Mini Kits (Qiagen, Switzerland) and reverse transcription was carried out using Superscript III RT (Invitrogen, Switzerland) according to the manufacturer`s instructions. qRT-PCR were performed using Rotor-Gene SYBR Green PCR Kit SYBR (Qiagen, Switzerland) and primers for Oct4, Sox2, Nanog, Rex1, and Dppa3 (Microsynth, Switzerland). Reactions were run in triplicates and using standard conditions on a Rotor-Gene Q (Qiagen, Switzerland).
- For detecting **Alkaline Phosphatase** (AP) activity, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Switzerland), washed with PBS and later with alkaline buffer for 10 minutes. Then alkaline phosphatase solution (10ml alkaline buffer containing 45µl NBT and 35µl BCP) was added and the reaction was stopped with TRIS-EDTA for 15 seconds. Stainings were analyzed with DMIL LED fluorescence microscope (Leica, Germany) and the Leica software (Leica, Germany).
- For **immunofluorescence**, hiPSCs were cultured on a 3.5cm dish, fixed with 4% paraformaldehyde, and incubated over night at 4°C with primary antibodies (antibodies are listed in Supplemental table 2). Staining was visualized with secondary antibodies (Supplemental table 2) for 1 hour incubation. Following, stainings were analyzed with DMIL LED fluorescence microscope and the Leica software.

- **In-vitro embryoid body formation**: HiPSCs were cultured in suspension in hiPSC medium without bFGF to form embryoid bodies (EB). After 3 weeks EB were harvested and RNA isolated for gene expression or plated for further differentiation (primer are listed in Supplemental table 1).
- **Teratoma assay:** $2x10^5$ undifferentiated, MEF free hiPSC were injected subcutaneously into one dorsal flank of NOD.CB17-Prkdc^{scid} mice. Teratomas were analysed microscopically (histology) after 10-12 weeks in vivo. The veterinary office of the Canton Zurich, Switzerland approved all animal experiments (ZH-171/2014).

3.2.3 Differentiation of hiPSCs

HiPSC were separated from MEF and cultured in suspension for 7 days in ultra-low attachment dishes (Corning, USA) in hiPSC medium without bFGF to form EB, which were then plated for further differentiation into SMCs and ECs

3.2.3.1 Smooth muscle cells

For muscle differentiation, attached EB were culture in high glucose DMEM (Sigma, Switzerland), 10% FBS (Sigma, Switzerland), 5% horse serum (Life Technologies, Switzerland), 100mM nonessential amino acids, 100mM 2-mercaptoethanol, 1mM L-glutamine, 50 U/ml penicillin/streptomycin. After 14 days medium was changed to Smooth Muscle Growth Medium-2 (SmGM-2) (Lonza, Switzerland) and further cultured for 14 days. All differentiated cells were fixed for immunofluorescence, lysed for total RNA extraction or further cultured for TE (antibodies and primers are listed in Supplemental tables 1 and 2). As a positive control primary human aortic SMC were commercially obtained (CC-2571;Lonza, Switzerland).

3.2.3.2 Endothelial cells

The differentiation of hiPSCs into ECs was induced according to previously reported protocol with some modifications [35]. Briefly, hiPSC were cultured in suspension for 10 days in a medium consisting of DMEM, 20% FBS, 2mM L-glutamine, 110uM 2-mercaptoethanol, 100uM NEAA, 50ng/ml ascorbic acid, 125U/ml penicillin, 125mg/ml streptomycin. EBs were dissociated into single cells with 2 mg/ml collagenase B (Roche, Switzerland) for 2 hours then Cell Dissociation Buffer (Invitrogen, Switzerland) for 15 min at 37°C shaking at 1,100 rpm. VE-cadherin+ cells were isolated by magnetic bead sorting (Miltenyi Biotec, USA). Isolated hiPSC-derived ECs were plated at 20,000 cells/cm^2 on fibronectin coated dishes (Corning, USA) in endothelial cell medium (EGM-2; Lonza, Switzerland). All differentiated cells were either fixed for immunofluorescence, or lysed for total RNA extraction or further cultured for TE (antibodies and primers are listed in Supplemental table 1 and 2). Moreover, as a positive control ECs were isolated from human umbilical cord with informed consent according to the cantonal ethics commission of Zurich, Switzerland [KEK-ZH-2009-0095].

3.2.5 Tissue Engineering

3.2.5.1 Scaffold fabrication

Vessels were fabricated from non-woven polyglycolic acid (PGA) meshes (thickness 0.5 mm; specific gravity 70 mg/cm3; Cellon, Luxembourg) and coated with 1.75% poly-4-hydroxybutyrate (P4HB; TEPHA, Inc., USA) by dipping into a tetrahydrofuran (THF) solution (Sigma-Aldrich, Switzerland). After solvent evaporation and vacuum drying overnight, the scaffolds were placed into a 70% EtOH (Sigma-Aldrich, Switzerland) for 30 min to obtain sterility, followed by two washing cycles with PBS (Sigma-Aldrich, Switzerland). Thereafter, scaffolds were pre-incubated in SmGM-2 with supplements for 12–24 hours to facilitate cell attachment.

3.2.5.2 Cell seeding

HiPSCs-derived SMCs were seeded onto scaffolds using $1.0*10^6$cells/cm^2. Fibrinogen (Sigma-Aldrich, Switzerland) (10 mg/mL of active protein) and thrombin (Sigma-Aldrich, Switzerland) were prepared, used and titrated to an optimal clotting time of approximately 30 sec by adapting the concentration of fibrinogen. The cells were resuspended in a fibrinogen-thrombin co-solution. After static incubation of seeded constructs in SmGM-2 (with supplements and 0.9mM of L-ascorbic acid-2-phosphate (Sigma-Aldrich, Switzerland)) for 7 days, they were either placed into a bioreactor under pulsatile flow for additional mechanical stimulation via shear stress (dynamic condition) or kept under static conditions. The pulsatile flow was directed through the inner lumen and mimicked the native cardiovascular environment with an constant flow over time (2.5ml/min). After 21 days of culture, hiPSCs-derived ECs ($1.0*10^6$cells/cm^2) were seeded into the lumen of TEVGs and kept for additional 3 days under static culture. The constructs were harvested after 24 days of culture and immediately processed for immunohistochemistry and quantitative tissue analysis.

3.2.6 Qualitative Tissue Analysis

For immunohistochemical analysis of TEVGs, 5µm sections derived from blocks of formalin-fixed, paraffin-embedded tissue were mounted on glass slides (SuperFrost Plus, Menzel Gläser,Germany), deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) or Masson Trichrome using standard histological techniques. All sections were analyzed using a Mirax Midi BF slide scanner and processed using MIRAX viewer (Zeiss, Germany). In addition, immunofluorescence for αSMA and vWF were performed by IHC-Service Lab, University Hospital Zurich, Switzerland. Images were captured with a DM6000B fluorescence microscope (Leica, Germany) and processed using the Leica software (Leica, Germany).

3.2.7 Quantitative Tissue Analysis

TEVGs (n = 4 per clone) were minced, lyophilized, and analyzed using biochemical assays for total deoxyribonucleic acid (DNA) content as an indicator for cell number, hydroxyproline (HYP) content as an indicator for collagen, as well as for glycosaminoglycan (GAG) content. All samples of cell constructs were

digested in papain (Sigma-Aldrich, Switzerland) solution (300 µg/mL in PBS with 5mM EDTA) (Sigma-Aldrich, Switzerland) and 5mM cysteine (Sigma-Aldrich, Switzerland) at 65°C for 16 hours. For measuring the cellularity of the constructs, the DNA amount was quantified according to manufacturer's protocol (Life Technologies, Switzerland, No. P11496). The GAG content was determined using a modified version of the protocol described by [36], as previously described, and a standard curve prepared from chondroitin sulfate from shark cartilage (Sigma-Aldrich, Switzerland). HYP was determined with a modified version of the protocol provided by [37]. Remnants of the co-polymers matrices were visualized using polarization microscopy.

3.2.8 Statistical analyses

Quantitative data are presented as mean ± standard deviation. Quantitative tissue analysis were statistically evaluated using an unpaired students-t-test and p-values $p<0.05$ were considered statistically significant. Post-hoc correction was performed using the Bonferroni method where applicable. Kolmogorov-Smirnov normality test was used to confirm that the datasets were normally distributed ($p>0.05$). All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software Inc., USA).

3.3 Results

3.3.1 Characterization of human PBMC derived iPSCs

PBMCs were isolated from human peripheral blood using Ficoll-Paque method and reprogramming into hiPSCs was induced according to previously reported protocol [25] by simultaneous transduction of viral vectors containing Oct4, Sox2, c-Myc and Klf4. After about 30 days, embryonic stem like colonies started to appear. Colonies displaying the characteristic morphology of human ESCs (Figure 1A) could be picked and expanded.

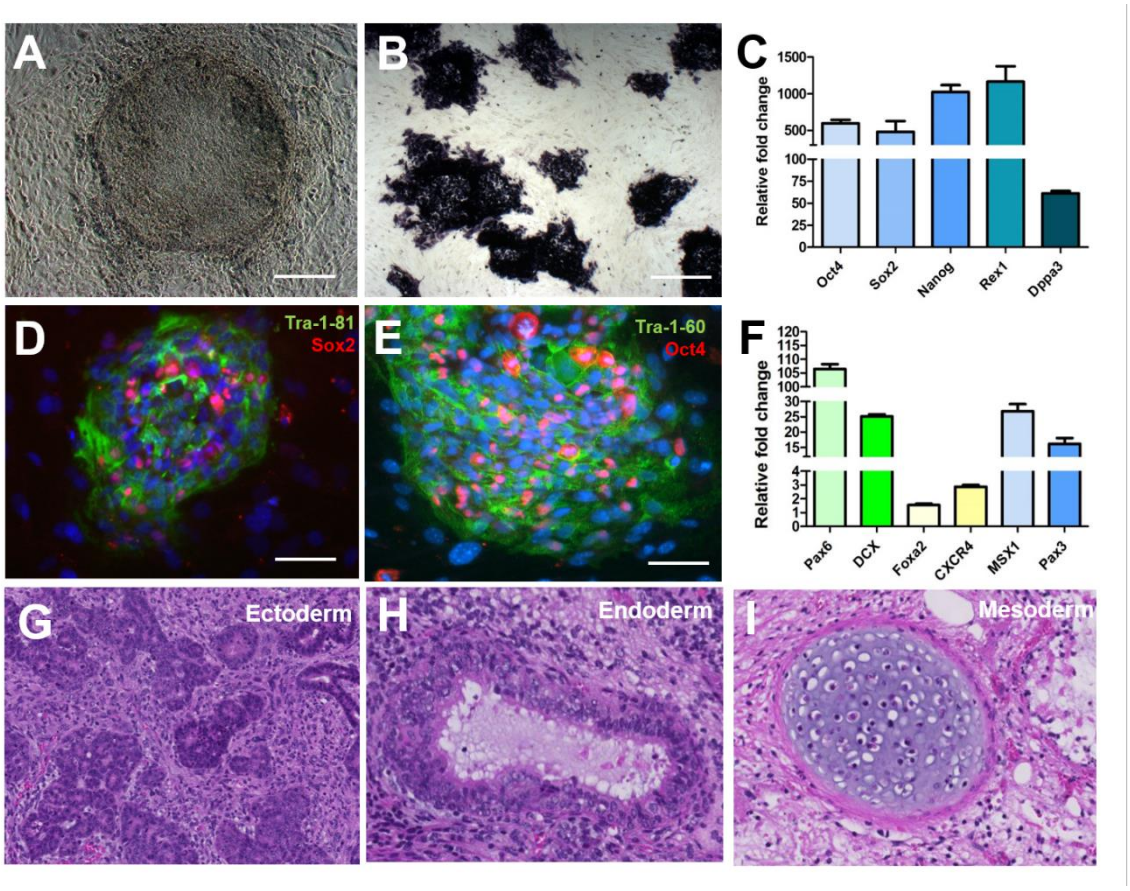

Figure 1: Characterization of human PBMC derived iPSCs. A) Morphology of hiPSC colony in culture. B) Alkaline phosphate staining of cultured hiPSCs C) Quantitative RT-PCR assay for expression analyses of pluripotency markers Oct4, Sox2, Nanog, Rex1, and Dppa3 in hiPSCs. Data are normalized with GAPDH and relative to PBMC. D-E) Immunostaining for pluripotency markers Oct4, Sox2, Tra-1-60 and Tra-1-81 in one representative iPSC line. (Dapi in blue) F) *In vitro* embryoid body (EB)-mediated differentiation of hiPSCs into three germ layers. Differentiated cells were analyzed by quantitative RT-PCR for the expression of endodermal (Foxa2 and CXCR4), ectodermal (Pax6 and DCX) and mesodermal (MSX1 and Pax3) lineage markers. Data are normalized with GAPDH and relative to undifferentiated iPSCs. G-I) H&E staining of teratomas arising of immunodeficient NSG mice. Teratoma containing neural tissues (ectoderm) (G), intestinal epithelium (endoderm) (H) and cartilage (mesoderm) (I). (Scale bars: 200µm) All experiments were performed for four different clones - Data show results of one representative clone (clone3).

These colonies showed high AP activity (Figure 1B) and highly expressed the pluripotency associated transcription factors Oct4, Sox2, Nanog, Rex1, and Dppa3 (Figure 1C). All markers were upregulated compared to the original, non-reprogrammed PBMCs, confirming their transition to an undifferentiated state. Furthermore, the human specific pluripotency markers Tra-1-81 and Tra-1-60 (Figure 1D and 1E) were

also exclusively expressed in the hiPSC colonies. To assess the pluripotent potential of the generated hiPSC, cells were first differentiated *in vitro* into EBs. Analysis of the expression of early differentiation markers revealed differentiation into all three germ layers: endoderm (Foxa2 and CXCR4), ectoderm (Pax6 and DCX) and mesoderm (MSX1 and Pax3) (Figure 1F). In addition, hiPSCs were injected subcutaneously into NOD-SCID mice and teratomas were generated after 10-12 weeks. Histological analyses revealed that cell types characteristic for all three germ layers including ectoderm (neural epithelium), mesoderm (cartilage) and endoderm (intestinal epithelium) were present (Figure 1G-I). Taken together, these results confirm the pluripotent nature of the generated hiPSCs.

3.3.2 Characterization of human iPSC-derived smooth muscle and endothelial cells

We next investigated the ability of three generated hiPSCs clones to differentiate into SMCs and ECs, in order to use them later for vascular TE applications. Under muscle inducing culture conditions, hiPSCs gradually lost the expression of the pluripotency genes (Figure 2B) and gained the expression of the SMCs specific genes like αSMA, calponin and SMMHC (Figure 2A). Moreover, the hiPSC-derived SMCs showed similar marker profile when compared to primary human aortic SMCs for specific SMC markers at RNA and protein level (Figure 2A and 2C). Similarly, hiPSC were also able to differentiate into ECs when cultured under specific conditions. Three different clones differentiated into ECs showed high expression of the ECs markers CD31, von Willebrand factor (vWF) and endoglin (Figure 3A) and concomitantly downregulation of the pluripotency markers Oct4, Nanog, and Rex1 (Figure 3B). Moreover, hiPSC-derived ECs expressed markers including CD31, vWF and endothelial nitric oxide synthase (eNOS) comparable to human umbilical cord derived endothelial cells (Figure 3C). In conclusion, the obtained hiPSC-derived SMCs and ECs were proved to be pure populations of differentiated cells, which highly resembled native SMCs and ECs (Figure 2A and 3A).

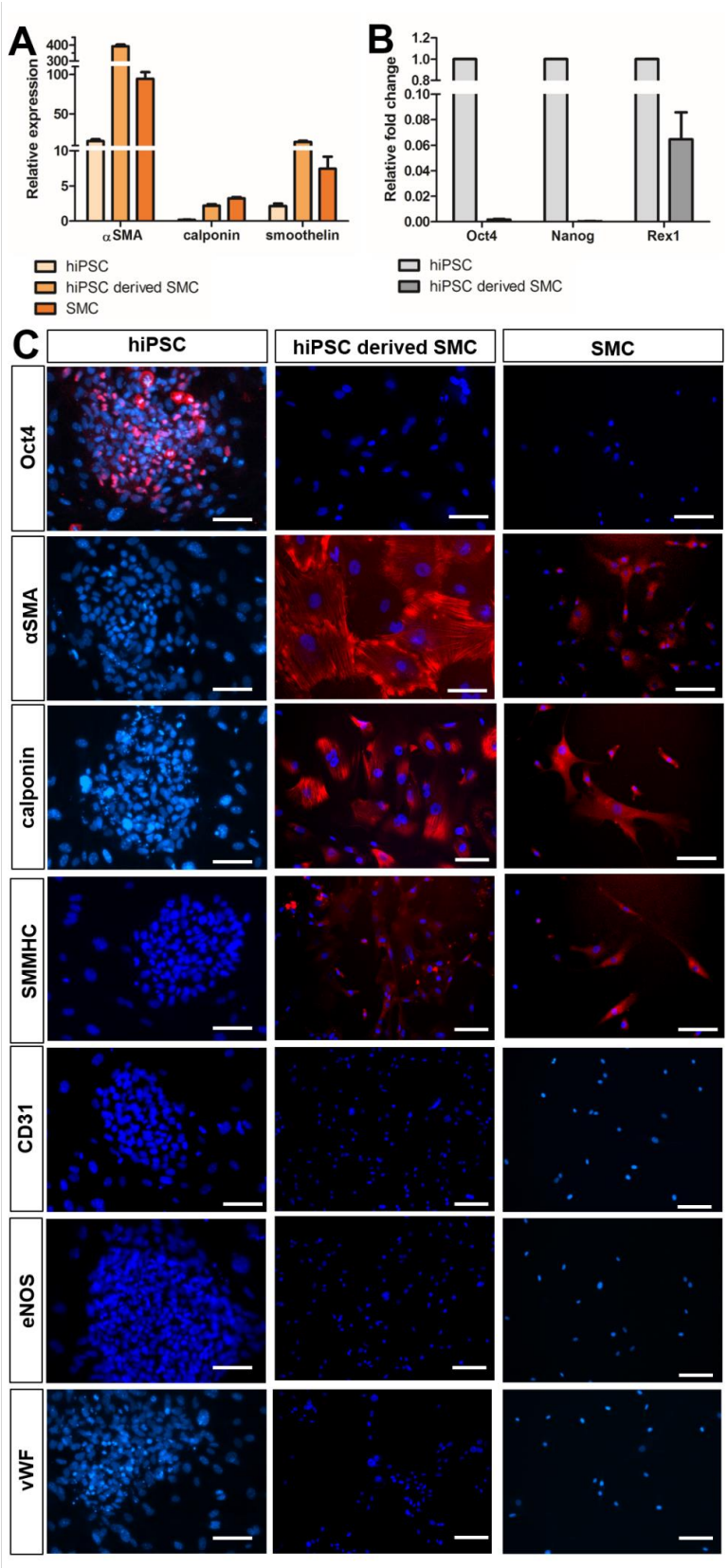

Figure 2: Characterization of differentiated smooth muscle cells (SMCs) from human iPSCs. Human iPSCs were differentiated into SMCs A) Quantitative RT-PCR assay for expression analyses of SMC markers αSMA, smoothelin, and calponin. Direct comparison between hiPSCs, hiPSC derived SMCs and primary SMCs. B) Quantitative RT-PCR assay for the expression of Oct4, Nanog, and Rex1 in hiPSC derived SMCs and hiPSC. Data were normalized with GAPDH and relative to the undifferentiated hiPSCs. C) Direct comparison of immunostaining for undifferentiated hiPSCs, hiPSC derived SMC, and primary SMCs. Following markers were tested: Oct4, α smooth muscle actin (αSMA), calponin, smooth muscle myosin heavy chain (SMMHC), CD31, von Willebrand factor (vWF), and endothelial nitric oxide synthase (eNOS). Dapi in blue and respective antibody in red. (Scale bars: 100µm) All experiments were performed with three different clones.

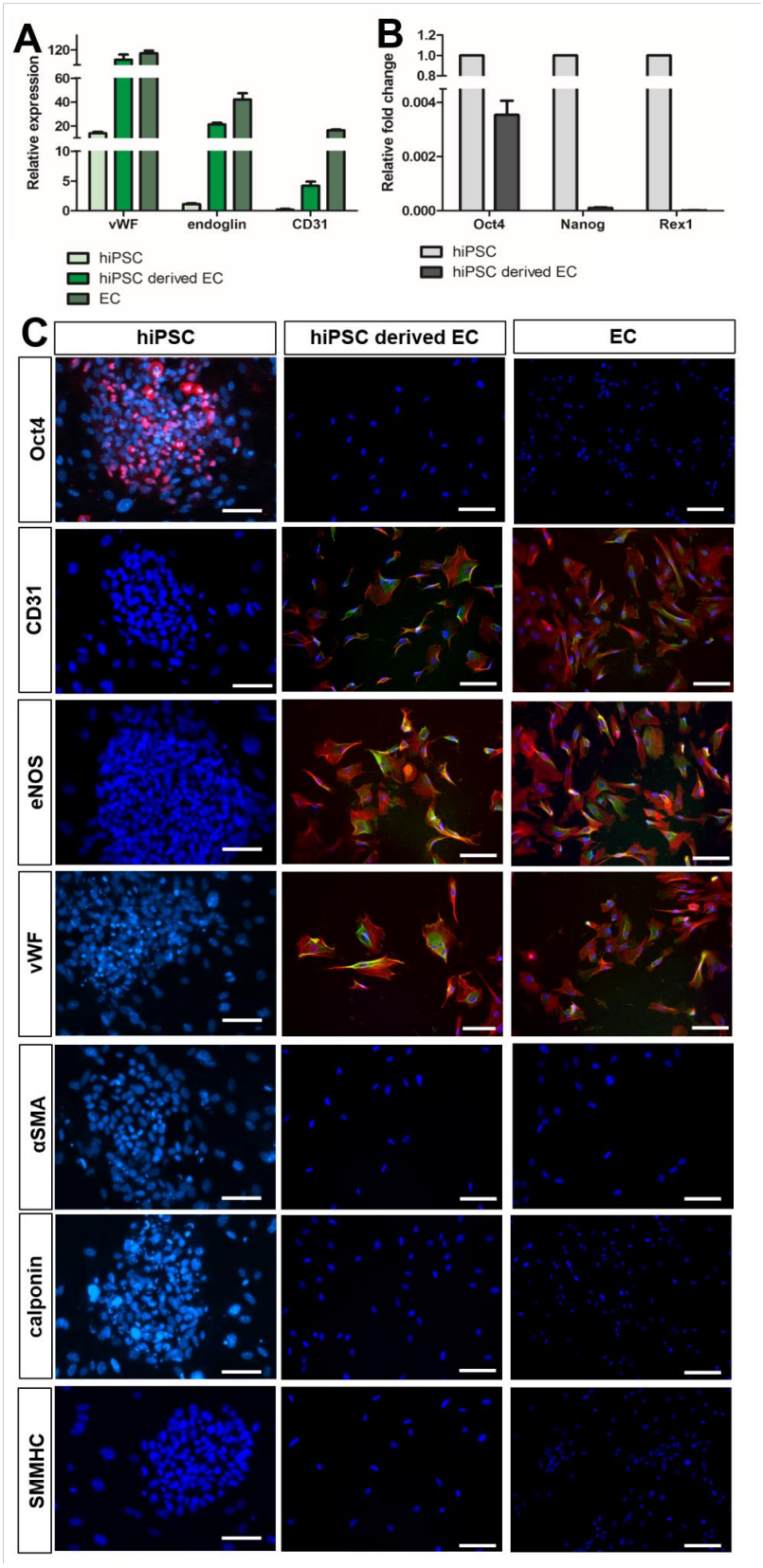

Figure 3: Characterization of differentiated endothelial cells (ECs) from human iPSCs. Human iPSCs were differentiated into ECs. A) Quantitative RT-PCR assay for expression analyses of EC markers von Willebrand factor (vWF), endoglin and CD31. Direct comparison between hiPSCs, hiPSC derived ECs and primary ECs. B) Quantitative RT-PCR assay for the expression of Oct4, Nanog, and Rex1 in hiPSC derived ECs and hiPSC. Data were normalized with GAPDH and relative to the undifferentiated hiPSCs. C) Direct comparison of immunostaining for undifferentiated hiPSCs, hiPSC derived EC, and primary ECs. Following markers were tested: Oct4, α smooth muscle actin (αSMA), calponin, smooth muscle myosin heavy chain (SMMHC), CD31, vWF, and endothelial nitric oxide synthase (eNOS). Dapi in blue, Phalloidin in red and respective antibody in green (Scale bars: 100µm) All experiments were performed with three different clones.

3.3.3 Qualitative and quantitative tissue analysis of TEVGs based on human iPSCs

Human iPSC-derived SMCs and ECs were seeded onto a PGA/P4HB scaffold and cultured under static or dynamic conditions. After 3 weeks (Figure 4A-B) the constructs were retrieved for histological analysis. H&E staining (Figure 4C-F) demonstrated formation of significant amounts of extracellular matrix (ECM) *in vitro*.

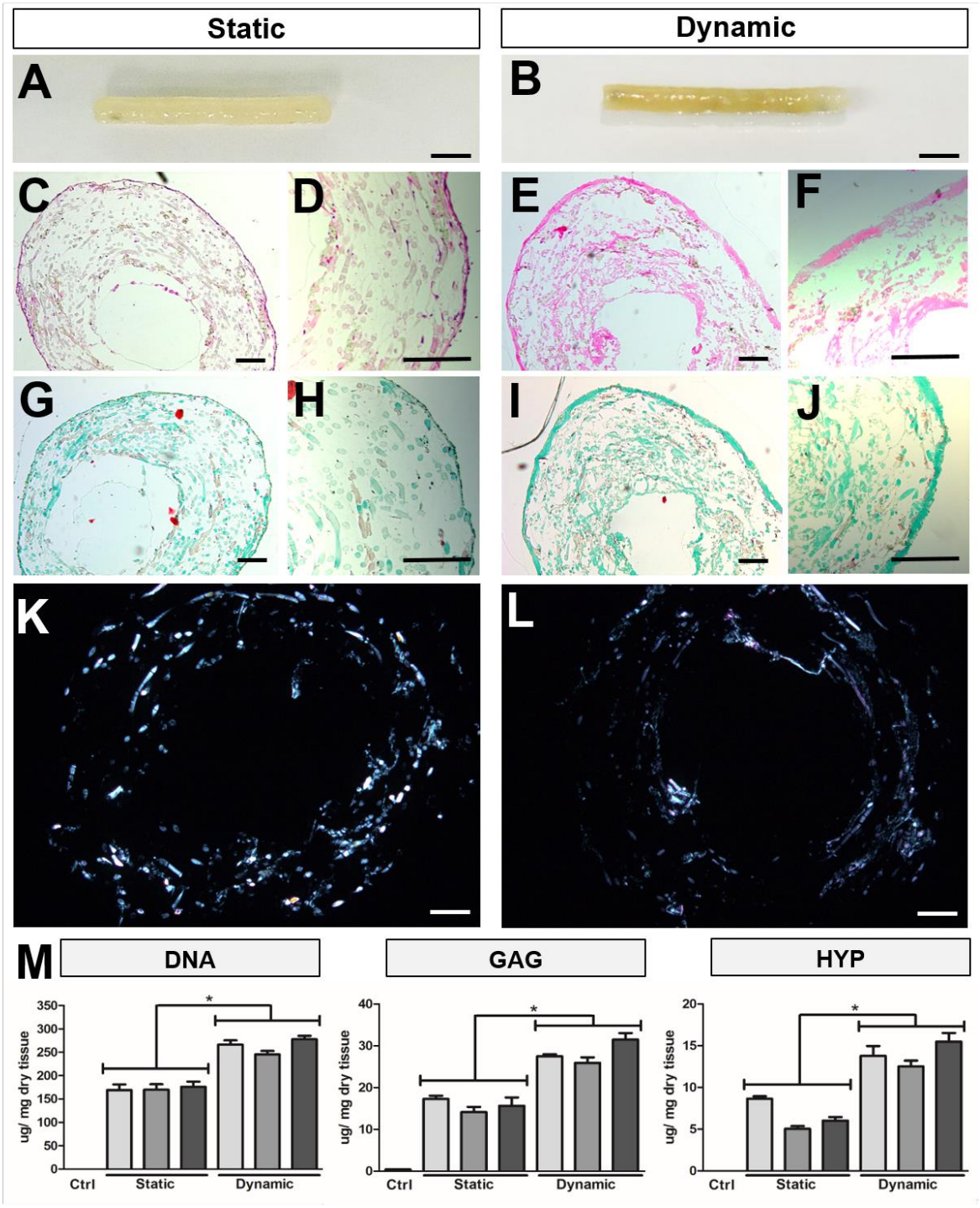

Figure 4: Qualitative and quantitative tissue analysis of TEVGs based on human iPSCs. Human iPSC-derived proliferative SMCs were seeded onto a PGA/P4HB scaffold and the scaffold construct were cultured under static or dynamic conditions. A-B) Macroscopic photos of TEVGs after 3 weeks of culture. C-F) H&E staining show tissue formation under both conditions. G-J) Masson's trichrome display collagenous extra cellular matrix formation after static and dynamic culture. K-L) Polarization microscopy revealed the presence of the initial scaffold matrix. M) Extracellular matrix analysis shows the amount of hydroxyproline (HYP), glycosaminoglycans (GAG), and the cell number (deoxyribonucleic acid, DNA) of TEVGs (n=4), which were statically or dynamically cultured (all values relative to control samples (ctrl) indicating biomaterials only). (Scale bars: 500µm)

TEVGs under static conditions (Figure 4C-D) displayed high cellularity and layered tissue on the outer vessel regions, while in the central part of the scaffold low cellularity and no visible formation of ECM were present. In contrast, TEVGs under dynamic conditions (Figure 4E-F) exhibited high cellularity and tissue formation on the outer as well as on the inner vessel regions. Nonetheless, low cellularity was demonstrated between these two layers compared to native controls. In order to visualize the deposition of collagen fibers Masson Trichrome staining was used (Figure 4G-J). Dynamically cultured TEVGs (Figure 4I-J) represented a better collagenous ECM deposition on the outer as well as on the inner vessel regions in comparison to TEVGs under static conditions (Figure 4G-H). In general, vascular grafts cultured under dynamic conditions showed a better cellular infiltration and tissue formation in comparison to static culture. Polarization microcopy revealed the presence of initial scaffold matrix in the central part of the constructs (Figure 4K-L). However, in dynamic constructs the biomaterial remnants were more degraded (Figure 4L) compared to static constructs (Figure 4K). The co-polymer starter matrices showed no major remodeling in the central part of the constructs under static as well as dynamic conditions given the lack of tissue formation in this area of the constructs. PGA fibers were visible as elongated ellipses. In order to quantify the composition of ECM, TEVGs were biochemically analyzed by using assays for HYP, GAG, and the cell number (DNA) (Figure 4M). Generally, all three hiPS cell lines (color pattern) displayed similar amounts of HYP, GAG and DNA for respective conditions with no significant differences detectable indicating lack of interindividual differences. Overall dynamic conditions (n=4) showed a significantly higher expression of DNA ($p<0.05$), GAG ($p<0.05$), and HYP ($p<0.05$) in comparison to static conditions (n=4). These findings underline the histological observations.

3.3.4 Immunofluorescence of TEVGs

To determine the cellular phenotypes in the TEVGs derived from hiPSCs, cells were stained for αSMA and vWF. Immunofluorescence stainings of TEVGs under static as well as dynamic conditions demonstrated layered tissue architecture with an αSMA positive layer located in the interstitium and a thin luminal layer of vWF positive cells comparable to native vessels (Figure 5). In general, the αSMA positive layer of TEVGs are thinner in comparison to native tissue. The majority of cells stained positive for αSMA were located at the outer layer of the statically cultured TEVG. In contrast to the static culture, dynamically cultured TEVG show a thicker αSMA positive layer.

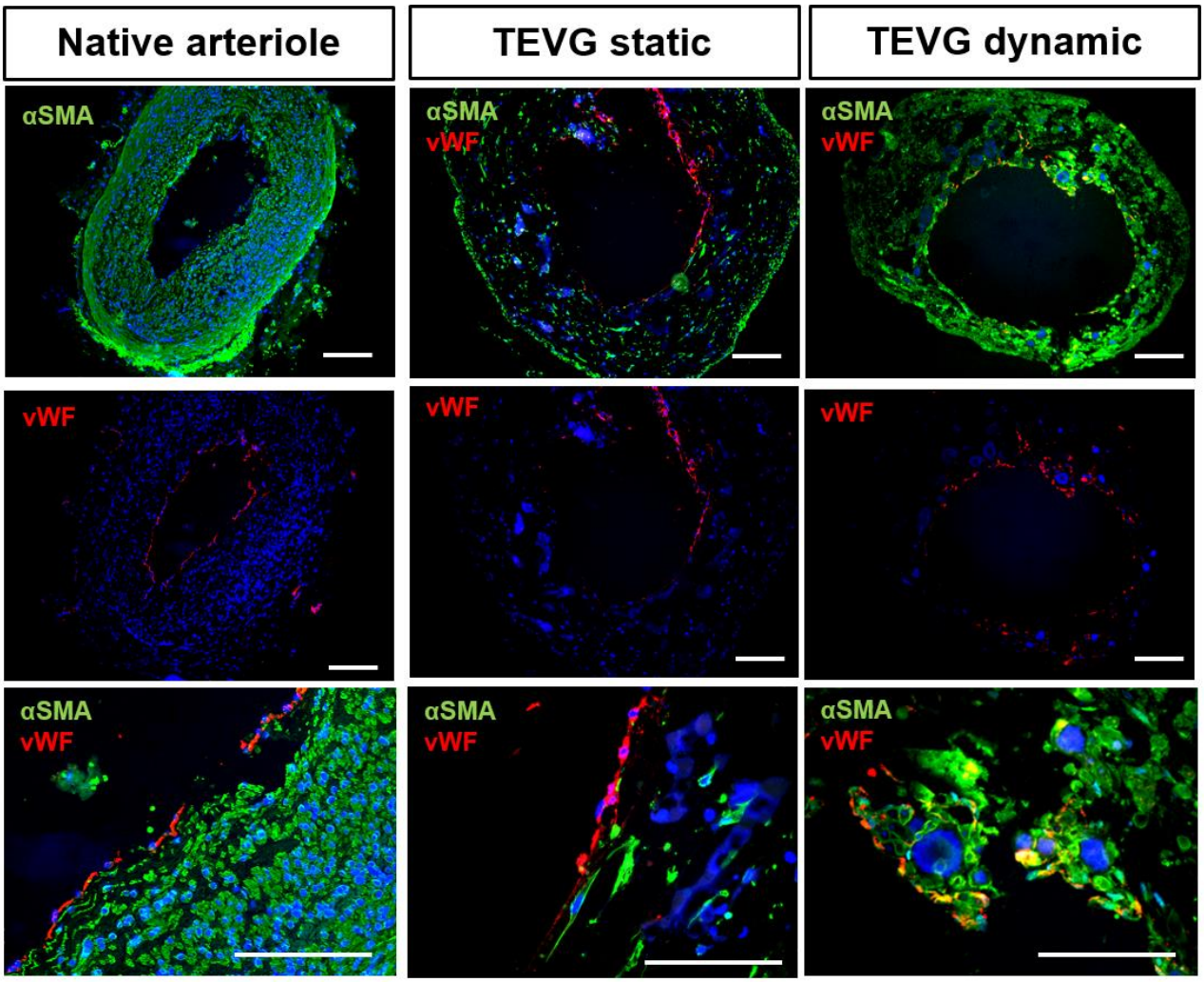

Figure 5: Comparative immunohistochemical anaylsis of tissue engineered vascular grafts (TEVGs) based on human iPSCs versus native arteriole. Immunohistological analyses of TEVGs under static and dynamic conditions (n=3) revealed positive expression of the smooth muscle cell marker alpha smooth muscle actin (αSMA) in the interstitium (green) and a thin luminal positive layer of endothelial cell marker von Willebrand factor (vWF) (red) (Dapi in blue). (Scale bars: 100µm)

3.4 Discussion

In vitro engineering of small diameter native-like vascular grafts is still demanding as poor haemocompatibility of TEVGs often leads to thrombosis and stenosis when implanted *in vivo* [5]. Therefore, creating a functional biomaterial that closely mimics the native layered architecture of blood vessels would represent a major step towards the functional replacement of disease vascular structures.

The current study is the first report showing the possibility to employ human PBMC-derived iPSCs to generate synthetic scaffold-based TEVGs, which contained a αSMA-positive layer in the graft interstitium as well as a thin luminal layer of vWF-positive cells comparable to native vessels. Importantly, given the ease of access, this PBMC-based approach may open up the possibility to generate native-like endothelialized 'customized' TEVGs i) to study complex human diseases *in vitro* not only enabling better understanding of the pathophysiology, but ii) ultimately also allowing for assessment of novel therapeutic approaches in the future.

Our data highlights the possibility to reprogram PBMCs to hiPSCs and make use of these cells for engineering small diameter vascular graft. The use of PBMCs has clear advantages compared to skin fibroblasts. Previously, the most common source to generate hiPSCs has been skin fibroblasts [18, 20]. Exposure of the dermis to ultraviolet (UV) light increases the risk for chromosomal aberrations [21], further, fibroblast cells need to expand for several passages *in vitro* and make it a cumbersome cell source for reprogramming. In addition, taking skin biopsies usually results in scar tissue formation and might not be feasible for non-therapeutic disease modeling settings or in volunteers. Beyond that, skin represents a non-reconstituting organ not providing unlimited supply to a certain donor tissue, implying that repetitive cell harvest seems difficult. In contrast, peripheral blood is a constantly reconstituting source of patient tissue that is not exposed to UV radiation. However, it represents a low invasive and well established cell source – a cell source one could use for large scale, repetitive isolation of sterile, viable, autologous, cellular material from patients as well as voluntary donors.

Our small diameter vascular graft displaying a layered tissue architecture similar to the native structure present in human vessels based on reprogramming PBMCs into hiPSC. These human PBMC-derived iPSCs were hence successfully differentiated into SMCs and ECs for their use in TE. The differentiated SMCs and ECs were seeded onto biodegradable polymer scaffolds and cultured under static or dynamic conditions in a bioreactor for inducing vascular tissue formation *in vitro*.

Various reprogramming techniques have been established during last years for generating hiPSCs [22]. Currently, four main categories can be subdivided into: integration-defective viral delivery, episomal delivery, RNA delivery and protein delivery [22]. The original method of reprogramming murine fibroblasts by Yamanaka utilized retroviral transduction of Oct4, Sox2, Klf4, and c-Myc into MEF [18]. As lentiviruses, unlike retroviruses, can infect non-dividing and proliferating cells, this approach became the most preferred

reprogramming approach. In particular, for clinical applications reducing genomic modifications to prevent insertional mutagenesis seems important, that is why more and more non-integrative or semi-integrative approaches have been developed. For example, our group recently established reprogramming of human cells into iPSC using a transgene-free approach [23]. The present study successfully achieved hiPSC reprogramming by simultaneously expressing the Yamanaka factors lentiviral vectors. Notwithstanding, in order to obtain cells suitable for clinical application, transgene-free hiPSCs need to be generated to avoid transgene reactivation, altered gene expression and misguided differentiation. Thus, future studies will establish PBMC-derived patient-specific, integration-free hiPSC and differentiate them into SMCs and ECs for TE applications.

In this study, hiPSC-derived TEVGs were generated under static and dynamic culture conditions. In general, vascular grafts cultured under dynamic conditions showed histologically an increased cellularity, tissue formation and scaffold degradation in comparison to static culture. In addition, dynamically cultured TEVGs expressed a significantly higher amount of DNA, GAG and HYP than static cultured TEVGs. It has been shown that mechanobiological interactions between cells and scaffolds can crucially influence cell behavior [24]. Wang et al. also showed an enhanced cell proliferation under dynamic three-dimensional (3D) culture compared with conventional static two-dimensional (2D) and 3D cell culture conditions [25]. In the field of vascular tissue engineering, a study using cyclic mechanical loading was reported by Niklason and colleagues [26]. In this study, tubular meshes of PGA were seeded with adult bovine aortic smooth muscle cells and placed around distensible silicone tubes for 8 weeks [26]. Finally, TE arteries were implanted in miniature swine, with patency up to 24 days.

The long-term patency of small-diameter vascular grafts is still a great challenge in the field of cardiovascular TE research. Early luminal thrombosis represents the major cause of vessel occlusion. A possible solution should be the coating of the grafts with antithrombogenic materials, such as heparin [27] or ethylene oxide [28], but the results were not satisfactory. The vascular TE approach has been introduced to generate vascular grafts with functional endothelium potentially enabling long-term patency [29]. This project showed the production of hiPSC-derived TEVGs, which contained an αSMA-positive layer in the interstitium and a thin luminal layer of vWF-positive cells comparable to native vessels. Gui et al. also observed thrombus formation in some samples, possibly due to the lack of endothelial cell coating of vascular grafts before implantation [30]. This underlines the impact of a functional endothelial cell layer in TEVGs. Future efforts will focus on the implantation of our TEVGs also to investigate the *in vivo* functionality and patency of human PBMC-iPSC-based TEVGs compared to mature vascular cell-derived constructs.

In the past, murine [31] and hiPSCs [32] were differentiated into SMCs and used to construct a TEVG for subcutaneous implantation for 2 weeks. Furthermore, Gui et al. implanted a hiPSC-derived TEVG into nude rats as abdominal aorta interposition for 2 weeks [30]. These TEVG contained α-SMA and SMMHC-positive cells [30]. So far, these studies are based on the reprogramming of human fibroblasts. Recently, Hu et al. established integration-free reprogrammed hiPSC from PBMCs and then differentiated the cells into

mesoderm-originated cardiovascular progenitor cells (CVPCs) [33]. Subcutaneous implantation of CVPCs seeded on a disc shaped scaffold led to *in vivo* lineage specification [33]. Nevertheless, there is no TEVGs based on SMCs and endothelial cells (ECs) differentiated from PBMC-derived hiPSC available yet.

Taken together our data show for the first time that it is possible to employ pre-differentiated human ECs and SMCs generated with PBMC-derived hiPSCs to produce vascular grafts comparable to native vessels.

Conclusion

Human iPSCs generated from PBMCs is a promising cell source for TE as they are easily accessible and may thus serve as a versatile platform also involving disease modeling applications. In addition to solving ethical concerns related to the use of blastocyst-derived ESC, the use of hiPSCs for the generation of therapeutic cells may avoid the requirement for post-transplant immunosuppression. Upon appropriate differentiation, these cells can then be used to study normal and pathologic human tissue development *in vitro*, enabling new insights into disease pathology as well as the development of novel therapeutic agents and patient-specific cellularized replacements. In summary, our study established an efficient approach towards generating patient-specific TEVGs based on human PBMC-iPSC-derived SMCs and ECs, containing a native-like layered vessel architecture.

Acknowledgments

The authors would like to thank Marko Canic and Ursula Steckholzer for their technical help and assistance. In particular, we would like to thank Gustavo Mostoslavsky from Boston Medical Center, who provided the vector and the knowledge for reprogramming PBMC into iPSC. The research leading to these results has received funding from the Swiss National Science Foundation (Project 310030_143992), Forschungskredit Candoc of the University of Zurich, Forschungskredit Postdoc of the University of Zurich, the Foundation for Research in Science and the Humanities at the University of Zurich and the Alfred and Anneliese Sutter-Stöttner-Foundation.

3.5 Supplementary Data

Gene	Forward Primer	Reverse Primer
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCCTGTTGCTGTA
Dppa3	GCTCACCGAAGAAAATTCCCG	GTTTGTTGGTCTTCTTTCATGCGTA
Nanog	ATACCTCAGCCTCCAGCAGA	GAGGCCTTCTGCGTCACAC
Oct4	GAGAAGCTGGAGCAAAACCC	ACCTTCCCAAATAGAACCCCC
Sox2	AACCAGCGCATGGACAGTTA	GACTTGACCACCGAACCCAT
Rex1	CATCGCTGAGCTGAAACAAATG	CTGCAGGTCTTGGCTTGACT
FOXA2	GGAGCAGCTACTATGCAGAGC	CGTGTTCATGCCGTTCATCC
CXCR4	ACTACACCGAGGAAATGGGCT	CCCACAATGCCAGTTAAGAAGA
Pax6	TGGGCAGGTATTACGAGACTG	ACTCCCGCTTATACTGGGCTA
DCX	TCCCGGATGAATGGGTTGC	GCGTACACAATCCCCTTGAAGTA
MSX1	ACACAAGACGAACCGTAAGCC	CACATGGGCCGTGTAGAGTC
Pax3	AGCTCGGCGGTGTTTTTATCA	CTGCACAGGATCTTGGAGACG
Calponin	CCTCTGCTCACTTCAACCGA	TTCTGGGCCAGCTTGTTCTTA
Smoothelin	CCTACCCTGGGTTTGAATTCTC	GAGTGCAGCCAGTTCTCCTT
αSMA	GTGTTGCCCCTGAAGAGCAT	GCTGGGACATTGAAAGTC
CD31	GAAATGTCCAGGCCAGCAGT	ATCTGCTTTCCACGGCATCA
vWF	GACGCCATCAACACCGAGTT	CTTTGTCGTTGGTTAGCTGGT
Endoglin	TGCACTTGGGATACAATTCCA	AGCTGCCCACTCAAGGATCT

Supplemental Table 1: Primers list

Primary antibody	**Company**	**Dilution**
anti-OCT4 (C52G3)	Cell Signaling, USA	1:200
anti-SOX2 (D6D9)	Cell Signaling, USA	1:200
anti-Tra-1-60 Biotin	eBioscience, Austria	1:200
anti-Tra-1-81 Biotin	eBioscience, Austria	1:200
Anti αSMA (1A4)	Abcam, United Kingdom	1:200
Anti calponin (HCP)	Sigma-Aldrich, Switzerland	1:200
Anti SMMHC (AB53219)	Abcam, United Kingdom	1:200
Anti CD31 (JC70A)	Dako, USA	1:200
Anti eNOS	Abcam, United Kingdom	1:200
Anti vWF	eBioscience, Austria	1:200
Phalloidin	Life Technologie, Switzerland	1:50
Dapi	Sigma-Aldrich, Switzerland	1:2000
Secondary antibody	**Company**	**Dilution**
anti rabbit Alexa Fluor 594	Invitrogen, USA	1:1000
anti mouse Alexa Fluor 594	Invitrogen, USA	1:1000
anti mouse Alexa Fluor 488	Invitrogen, USA	1:1000
anti rabbit Alexa Fluor 488	Invitrogen, USA	1:1000

Supplemental Table 2: Antibodies list

3.6 References

1. Mozaffarian, D., et al., *Heart disease and stroke statistics--2015 update: a report from the American Heart Association.* Circulation, 2015. **131**(4): p. e29-322.
2. Isenberg, B.C., C. Williams, and R.T. Tranquillo, *Small-diameter artificial arteries engineered in vitro.* Circ Res, 2006. **98**(1): p. 25-35.
3. Chard, R.B., et al., *Aorta-coronary bypass grafting with polytetrafluoroethylene conduits. Early and late outcome in eight patients.* J Thorac Cardiovasc Surg, 1987. **94**(1): p. 132-4.
4. Kannan, R.Y., et al., *Current status of prosthetic bypass grafts: a review.* J Biomed Mater Res B Appl Biomater, 2005. **74**(1): p. 570-81.
5. Veith, F.J., et al., *Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal arterial reconstructions.* J Vasc Surg, 1986. **3**(1): p. 104-14.
6. Kidane, A., et al., *Surface modification with PEO-containing triblock copolymer for improved biocompatibility: in vitro and ex vivo studies.* J Biomater Sci Polym Ed, 1999. **10**(10): p. 1089-105.
7. Devine, C., B. Hons, and C. McCollum, *Heparin-bonded Dacron or polytetrafluoroethylene for femoropopliteal bypass grafting: a multicenter trial.* J Vasc Surg, 2001. **33**(3): p. 533-9.
8. Shinoka, T. and C. Breuer, *Tissue-engineered blood vessels in pediatric cardiac surgery.* Yale J Biol Med, 2008. **81**(4): p. 161-6.
9. Bajpai, V.K. and S.T. Andreadis, *Stem cell sources for vascular tissue engineering and regeneration.* Tissue Eng Part B Rev, 2012. **18**(5): p. 405-25.
10. Weber, B., et al., *Engineering of living autologous human umbilical cord cell-based septal occluder membranes using composite PGA-P4HB matrices.* Biomaterials, 2011. **32**(36): p. 9630-41.
11. Cleary, M.A., et al., *Vascular tissue engineering: the next generation.* Trends in Molecular Medicine, 2012. **18**(7): p. 394-404.
12. Naito, Y., et al., *Vascular tissue engineering: towards the next generation vascular grafts.* Adv Drug Deliv Rev, 2011. **63**(4-5): p. 312-23.
13. Galmiche, M.C., et al., *Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway.* Blood, 1993. **82**(1): p. 66-76.
14. Kashiwakura, Y., et al., *Isolation of bone marrow stromal cell-derived smooth muscle cells by a human SM22alpha promoter: in vitro differentiation of putative smooth muscle progenitor cells of bone marrow.* Circulation, 2003. **107**(16): p. 2078-81.
15. Li, X., et al., *Revascularization of ischemic tissues by PDGF-CC via effects on endothelial cells and their progenitors.* J Clin Invest, 2005. **115**(1): p. 118-27.
16. Rodriguez, L.V., et al., *Clonogenic multipotent stem cells in human adipose tissue differentiate into functional smooth muscle cells.* Proc Natl Acad Sci U S A, 2006. **103**(32): p. 12167-72.
17. Choudhery, M.S., et al., *Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation.* J Transl Med, 2014. **12**: p. 8.
18. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
19. Sommer, A.G. *Generation of iPSC from Human Peripheral Blood*. 2011.
20. Lowry, W.E., et al., *Generation of human induced pluripotent stem cells from dermal fibroblasts.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 2883-8.
21. Ikehata, H., et al., *Analysis of mutation spectra in UVB-exposed mouse skin epidermis and dermis: frequent occurrence of C-->T transition at methylated CpG-associated dipyrimidine sites.* Environ Mol Mutagen, 2003. **41**(4): p. 280-92.
22. Gonzalez, F., S. Boue, and J.C. Izpisua Belmonte, *Methods for making induced pluripotent stem cells: reprogramming a la carte.* Nat Rev Genet, 2011. **12**(4): p. 231-42.
23. Slamecka, J., et al., *Non-integrating episomal plasmid-based reprogramming of human amniotic fluid stem cells into induced pluripotent stem cells in chemically defined conditions.* Cell Cycle, 2016. **15**(2): p. 234-49.

24. Mauck, R.L., et al., *Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels.* J Biomech Eng, 2000. **122**(3): p. 252-60.
25. Tang, Y., et al., *The combination of three-dimensional and rotary cell culture system promotes the proliferation and maintains the differentiation potential of rat BMSCs.* Sci Rep, 2017. **7**(1): p. 192.
26. Niklason, L.E., et al., *Functional arteries grown in vitro.* Science, 1999. **284**(5413): p. 489-93.
27. Laredo, J., et al., *Silyl-heparin adsorption improves the in vivo thromboresistance of carbon-coated polytetrafluoroethylene vascular grafts.* Am J Surg, 2003. **186**(5): p. 556-60.
28. Park, K., et al., *In vitro and in vivo studies of PEO-grafted blood-contacting cardiovascular prostheses.* J Biomater Sci Polym Ed, 2000. **11**(11): p. 1121-34.
29. L'Heureux, N., et al., *A completely biological tissue-engineered human blood vessel.* FASEB J, 1998. **12**(1): p. 47-56.
30. Gui, L., et al., *Implantable tissue-engineered blood vessels from human induced pluripotent stem cells.* Biomaterials, 2016. **102**: p. 120-9.
31. Xie, C., et al., *Three-dimensional growth of iPS cell-derived smooth muscle cells on nanofibrous scaffolds.* Biomaterials, 2011. **32**(19): p. 4369-75.
32. Wang, Y., et al., *Engineering vascular tissue with functional smooth muscle cells derived from human iPS cells and nanofibrous scaffolds.* Biomaterials, 2014. **35**(32): p. 8960-9.
33. Hu, J., et al., *Patient-specific cardiovascular progenitor cells derived from integration-free induced pluripotent stem cells for vascular tissue regeneration.* Biomaterials, 2015. **73**: p. 51-9.
34. Sommer, A.G., et al., *Generation of human induced pluripotent stem cells from peripheral blood using the STEMCCA lentiviral vector.* J Vis Exp, 2012(68).
35. Adams, W.J., et al., *Functional vascular endothelium derived from human induced pluripotent stem cells.* Stem Cell Reports, 2013. **1**(2): p. 105-13.
36. Farndale, R.W., D.J. Buttle, and A.J. Barrett, *Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue.* Biochim Biophys Acta, 1986. **883**(2): p. 173-7.
37. Huszar, G., J. Maiocco, and F. Naftolin, *Monitoring of collagen and collagen fragments in chromatography of protein mixtures.* Anal Biochem, 1980. **105**(2): p. 424-9.

Chapter 4

General Discussion

4.1 The optimal material for TE applications: Synthetic vs. natural

For tissue engineering applications, the expanded cells are seeded onto a biodegradable scaffold. The biomaterial mimics the biologic and mechanical function of the native ECM found in tissues in the body by serving as an artificial ECM. Generally, three classes of biomaterials have been investigated for engineering tissues: (i) naturally-derived materials, (ii) acellular tissue matrices, and (iii) synthetic polymers.

The present study aimed at a systematic, multimodal comparison of three frequently used synthetic polymers for the in vitro engineering of extracellular matrix based on PGA which is the most common used polymer for TE applications under static as well as dynamic conditions.

Due to their high durability and mechanical strength several synthetic polymers have been used in the attempt to produce scaffolds for TE applications, including PGA, P4HB, PLA, and PCL: Therefore, three human fibroblast cell lines were seeded on either PGA-P4HB, PGA-PLA or PGA-PCL patches. These patches were analyzed after 21 days of culture qualitatively by histology and quantitatively by determining the amount of DNA, GAG and HYP. We found that PGA-P4HB and PGA-PLA scaffolds stimulate tissue formation significantly higher than PGA-PCL scaffolds ($p<0.05$). Polymer remnants were visualized by polarization microscopy. The degradation products of PGA, PLA, and P4HB are non-toxic, natural metabolites and are eventually eliminated from the human body [1, 2]. The degradation rate of these polymers can be adjusted from several weeks to several years by altering crystallinity, initial molecular weight, and the copolymer ratio of lactic to glycolic acid [2]. In addition, biomechanical properties of the tissue engineered patches were determined in comparison to native tissue. PGA-PLA and PGA-P4HB TE patches show similar biomechanical properties. Both hybrid polymers are more ductile and flexible in comparison to PGA-PCL. Production conditions of synthetic polymers can be tightly regulated, thus, mechanical and physical properties are predictable and well-defined [3]. Taken as a whole, all PGA based scaffolds showed tissue formation after 3 weeks of culture and mechanical flexibility and strength.

In addition, synthetic polymer scaffolds are less expensive, better reproducible and may be stored off-the-shelf over longer time periods [4]. These characteristics make them an interesting raw material for scaffold fabrication. Nevertheless, their biocompatibility issues harbor also complications.

Natural polymers usually contain specific molecular domains that can promote and guide cells and can thus enhance biological interaction of the scaffold with the host tissue [5]. Natural scaffolds composed of ECM proteins are a promising alternative for tissue repair and regeneration. These types of scaffolds have been shown to support the interaction with the surrounding host tissue, to induce the deposition of cells and ECM, and to accelerate the process of angiogenesis [6]. However, producing scaffolds from natural materials with homogenous and reproducible structures is quiet challenging. Additionally,

these scaffolds show poor mechanical properties, which leads to a limited use [3]. In contrast, we showed that PGA-PCL represents a robust scaffold material.

Table1 summarizes the advantages and disadvantages of synthetic and natural scaffolds for TE. In order to enhance the biological capacity and to overcome the disadvantages of these individual biomaterials, groups have combined synthetic with natural polymers [7, 8]. Future studies will focus on an adaptation of the concentrations of the used synthetic polymers, also in combination with natural polymers for TE applications.

	Natural Polymers	Synthetic Polymers
ADVANTAGES	Biocompatible Non-toxic degradation Abundant in the natural environment	Easy processable Controlled synthesis Physical properties flexible Good mechanical properties
DISADVANTAGES	Weak mechanical properties Hard processable Undefined degradation rate	Possible toxic degradation products High production costs Low biocompatibility

Table1: Natural versus synthetic polymers

4.2 Technical advances in iPSC generation

In 2006, Yamanaka and Takahashi evaluated 24 genes thought to be relevant for pluripotency and introduced them into mouse fibroblast cells using retroviral transduction system [9]. Finally, they proved that only four genes are required to generate ESC-like cells. In order to translate the iPSC technology into clinical applications, suitable techniques of factor delivery and efficient identification and isolation of reprogrammed cells are essential. Therefore, significant efforts have been investigated in the field of iPSC generation and identification by several groups around the globe. Already one year after the first generation of iPSCs, Thomson et al. successfully reprogrammed human fibroblasts using a lentiviral system [10]. As lentiviruses, unlike retroviruses, can infect non-dividing and proliferating cells, this approach became the most preferred reprogramming approach. The present study successfully achieved hiPSC reprogramming by using lentiviral delivery. However, the use of integrating viruses as a delivery system could lead to undesired insertional mutagenesis in the target cells. In order to obtain cells suitable for clinical application, many approaches to generate transgene-free hiPSCs

have been employed, such as plasmids [11], Sendai virus [12], adenovirus [13], protein compounds [14], small molecules [15], and microRNAs [16].

Standardized protocols for general cell products are required for clinical use of iPSCs. The first culture condition for human pluripotent stem cells was established for maintaining human ESCs [17]. This medium contained several animal-derived products including mouse embryonic fibroblasts for feeder layers and fetal bovine serum (FBS) in culture medium. This culture system was successfully adapted to human iPSCs, and it maintained their pluripotency and self-renewal [18]. During the last years, removal of animal-derived feeder cells and chemically defined conditions for culturing hiPSCs have attracted growing attention. As an alternative for culturing hiPSCs without a feeder layer, Matrigel has been proven to be a useful option that allows stable culture of human pluripotent stem cells [19, 20]. Nevertheless, matrigel is derived from Engelbreth-Holm-Swarm mouse tumor, and thus his condition was not animal product-free [21]. Other xeno-free agents, for example recombinant proteins [22, 23] and synthetic polymers [24, 25] have been studied and used as feeder-free substitute for generation and culture of human pluripotent cells. Furthermore, to remove FBS that might cause the differentiation of human ESCs, knockout serum replacement (KSR) has now been established for maintaining human ESCs [26] and hiPSCs [18]. In addition, mTeSR1 medium was developed as a chemically defined medium for maintaining human pluripotent cells [27]. However, both KSR and mTeSR1 also contain animal-derived products and new media have now been developed as xeno-free media for maintaining human pluripotent stem cells, such as TeSR2 [28] and Essential E8 [22]. In our study, we have generated and maintained hiPSCs on a mouse feeder layer with ESC media containing KSR. Developing animal product-free conditions for iPSC generation is also an important practice for achieving safe therapies, thus, future studies will establish PBMC-derived patient-specific, integration-free hiPSC under xenogene-free conditions.

4.3 Equivalency of iPSCs and ESCs: Are iPSCs different from ESCs?

After the establishment of the first iPSCs, researchers were impressed by the remarkable similarity to ESCs for years [9]. ESCs and iPSCs share two key characteristics in common: (i) ability to self-renew and (ii) they can differentiate into cells from all three germ layers. During the last years, however, scientists started reporting more and more differences between iPSCs and ESCs [29, 30]. Analyses of genome-wide expression patterns and global histone modifications have demonstrated a high degree of similarity between ESCs and iPSCs [31-33]. Deng et al. first discovered differences in DNA methylation between both pluripotent stem cell types [34]. Later other groups reported differentially methylation as well, including BMP3 [35]. However, other studies showed that it is difficult to distinguish iPSCs from ESCs by gene expression or DNA methylation [36]. Interestingly, by a detailed examination how many clones were evaluated, a clear tendency was detected by Yamanka [37]. Studies with a relatively small

number of clones reported differences, whereas reports with many more clones found it difficult to distinguish between ESCs and iPSCs based on gene expression or DNA methylation [37].

Essential differences between these two pluripotent cell types have been reported for the differentiation ability. For instance, a reduced neuronal differentiation potential has been shown for 12 hiPSC lines [38]. ESC clones showed a differentiation efficiency of 90%, in contrast iPSC clones only differentiated with 10 to 50% efficiency [38]. Additionally, iPSCs derived from human and murine fibroblasts can differentiate into functional cardiomyocytes [39, 40]. However, iPSCs displayed a delayed and less efficient differentiation of beating cardiomyocytes compared with ESCs [41]. In addition, variation exists regarding the expression of genetic markers in iPSC-derived cardiac cells as compared to ESC-derived cardiomyocytes [41, 42]. We have generated hiPSCs from PBMCs and differentiated them into αSMA- and calponin-positive SMCs as well as ECs positive for CD31, vWF and eNOS that highly resembled native SMCs and ECs. The hiPSC-derived SMCs and ECs showed similar marker profile when compared to primary human aortic SMCs, respectively primary human ECs for specific markers at RNA and protein level. Although multiple differentiation protocols have been established, there is still a substantial variability in the efficiency of generating differentiated lineages among independent iPSCs andESCs [43].

4.4 Therapeutic potential of iPSC

Human iPSCs are a promising cell source in the field of regenerative medicine as they are easily accessible and may thus serve as a versatile platform also involving disease modeling applications. In addition to solving ethical concerns related to the use of blastocyst-derived ESCs. The use of hiPSCs allows for cell transplantation without the need for any immunosuppression or possible allogeneic tissue immune rejection given their autologous nature. Upon appropriate differentiation, these cells can then be used to study normal and pathologic human tissue development *in vitro*, enabling new insights into disease pathology as well as the development of novel therapeutic agents and patient-specific cellularized replacements. Figure 1 presents potential applications of hiPSCs.

4.4.1 Cell therapy

Since their discovery, iPSCs have captured the imagination of researchers and clinicians seeking to develop patient-specific therapies. Currently, several clinical trials evaluating stem cell therapy products are ongoing [44, 45]. Nevertheless, until today there has been only one clinical trial involving iPSC-derived cells to treat wet-type macular degeneration (AMD) [46]. Wet-eye AMD is associated with an abnormal growth of new blood vessels behind the retina, which causes the progressive damage of the retinal pigment epithelial (RPE) cells. Therefore, RPE sheets derived from the patient`s own iPSCs were transplanted into the right eve of a 70-year old female patient [46].

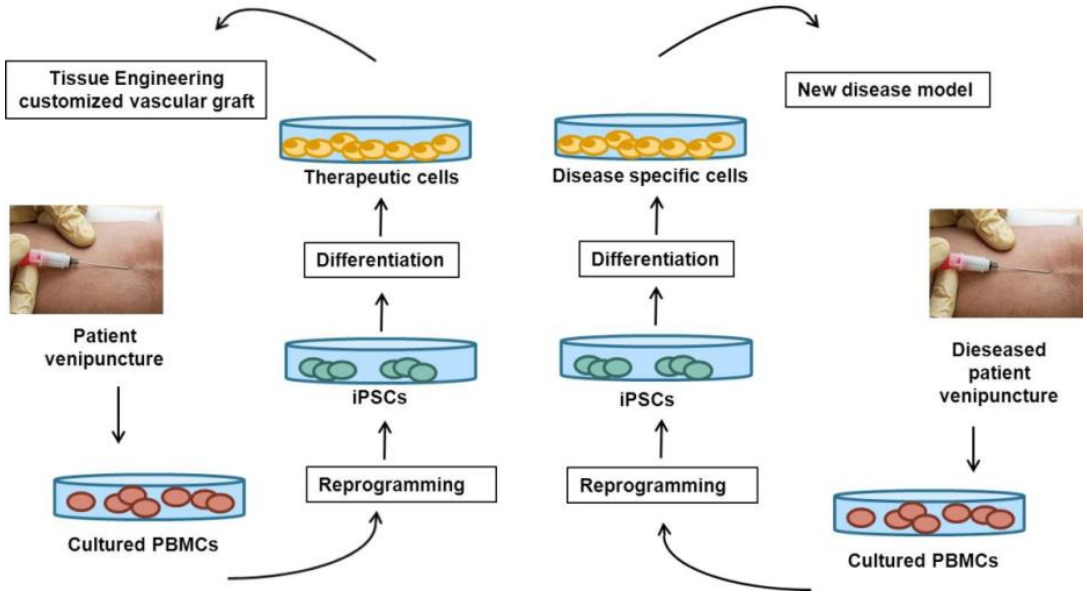

Figure 1. Applications for iPSCs.
iPSCs enable to develop new models to study human disease and represent a significant step towards patient-specific cell replacement therapies.

The therapy resulted in a stabilization of her visual acuity and stopping macular degeneration. However, the trial was stopped owing to two mutations detected in the RPE cells that were not present in the patient`s original fibroblast cells [47, 48]. These findings underline that there are still hurdles related to iPSC-based therapies that will need to be addressed before routine clinical applications can start. One issue is the risk of tumorigenicity from iPSCs, as well as, from ESCs [49]. During long culture periods pluripotent cell can accumulate karyotypic abnormalities and copy number variants [50]. Thus, it is even more important to carefully screen iPSC-derived products for risky genetically aberrations before clinical use. Interestingly, products differentiated from iPSCs have not been shown to generate teratomas [51, 52]. Thus, standardized protocols for differentiating hiPSCs with specific identity and cellular function are essential, as well as, the elimination of undifferentiated cells.

Another promising study suggest that the treatment of genetic disorders with iPSCs could be feasible. Already in 2007, Hanna et al. demonstrated the use of iPSC to rescue the defects seen in an animal model of sickle cell anemia [53]. Sickle cell anemia relies on a single point mutation in the hemoglobin gene. In this study, murine skin cells were reprogrammed into iPSCs and subsequently the disease-causing mutation was corrected by gene targeting [53]. The healthy progenitors were transplanted into anemic mice. Finally, the transplanted cells produced normal red blood cells and cured the disease [53]. In theory, this approach could be adapted to any disease in humans with a known mutation and that can be treated by cell transplantation.

In addition, autologous iPSCs might reduce the high costs and side effects related to the requirement of lifelong immunosuppression for allogeneic cell transplantation [54]. Furthermore, recent studies have shown that the differentiation of iPSC could lead to loss of immunogenicity [55, 56].

4.4.2 Disease modeling and Drug discovery

The study and treatment of many diseases, such as type I diabetes, Alzheimer`s disease, and Parkinson`s disease is limited by the availability of the affected tissue, as well as insufficient culture conditions to grow the cells. Therefore, iPSCs provide an unlimited source of proliferating cells and overcome the constraints of confined donor cell availability as well as limited proliferation capacity. The potential of using iPSCs for disease modelling *in vitro* and to generate new diagnostic / therapeutic approaches provide excellent platforms for studying human diseases in a dish. A growing number of researchers have employed iPSCs for modelling a variety of diseases, including diseases of the hematopoietic [57-59], neurological [60-62], endothelial [63], and cardiovascular [64, 65] system. They demonstrated the possibility of using patient-derived iPSCs for reproduction of human disease phenotypes and their potential application in the use of these cells for drug screening. Our study showed the generation of hiPSCs based on PBMCs and differentiation into αSMA- and calponin-positive SMCs as well as ECs staining positive for CD31, vWF and eNOS. These cells were seeded onto PGA-P4HB starter matrices and cultured under static or dynamic conditions to induce tissue formation *in vitro*. Resulting TEVGs showed abundant amounts of extracellular matrix, containing a αSMA-positive layer in the interstitium and a thin luminal layer of vWF-positive cells approximating native vessels. These results pave the way for developing autologous PBMC-derived hiPSC-based vascular constructs for therapeutic applications or disease modeling.

Another interesting approach is the use of hiPSCs for the production of organoids. Recently, Qian et al. generated brain organoids based on hiPSCs to clarify whether and how infection of the Zika virus in pregnant woman could lead to microcephaly during development of the fetus [66]. After exposure to the virus, it mainly infected neural stem cell progenitors leading to cell death and reduced proliferation [66]. These and other findings have helped to understand the mechanism of this disease.

The ultimate goal is to gain greater insight into mechanisms of human disease or to develop new and improved therapies or diagnostics. Although great advances have been made in terms of developing disease models in animals, such as transgenic mice, there are several limitations when focusing on the translation of the data generated with regards to human disease. There have already been drugs developed, which show therapeutic effects in rodent models, but are inefficient in the human situation. An example for this represents the field of the amyotrophic lateral sclerosis [67]. In contrast, when iPSCs were exposed to experimental drugs for specific diseases, the “symptoms” were partially alleviated in culture [33]. Therefore, disease modeling with human iPSCs represents an indispensable step for the development of therapies for human disease.

4.5 References

1. Chen, G.Q. and Q. Wu, *The application of polyhydroxyalkanoates as tissue engineering materials.* Biomaterials, 2005. **26**(33): p. 6565-78.
2. Koh, C.J. and A. Atala, *Tissue engineering, stem cells, and cloning: Opportunities for regenerative medicine.* Journal of the American Society of Nephrology, 2004. **15**(5): p. 1113-1125.
3. Asti, A. and L. Gioglio, *Natural and synthetic biodegradable polymers: different scaffolds for cell expansion and tissue formation.* Int J Artif Organs, 2014. **37**(3): p. 187-205.
4. Dijkman, P.E., et al., *Polymeric Starter Matrices for Cardiovascular Tissue Engineering.* Encyclopedia of Biomedical Polymers and Polymeric Biomaterials, 2015(CRC Press 2015): p. 1–25.
5. Nooeaid, P., et al., *Osteochondral tissue engineering: scaffolds, stem cells and applications.* J Cell Mol Med, 2012. **16**(10): p. 2247-70.
6. Laschke, M.W., et al., *Angiogenesis in tissue engineering: Breathing life into constructed tissue substitutes.* Tissue Engineering, 2006. **12**(8): p. 2093-2104.
7. Friess, W. and M. Schlapp, *Sterilization of gentamicin containing collagen/PLGA microparticle composites.* Eur J Pharm Biopharm, 2006. **63**(2): p. 176-87.
8. Wu, W., et al., *Engineering of human tracheal tissue with collagen-enforced poly-lactic-glycolic acid non-woven mesh: a preliminary study in nude mice.* Br J Oral Maxillofac Surg, 2007. **45**(4): p. 272-8.
9. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-76.
10. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells.* Science, 2007. **318**(5858): p. 1917-20.
11. Okita, K., et al., *A more efficient method to generate integration-free human iPS cells.* Nat Methods, 2011. **8**(5): p. 409-12.
12. Fusaki, N., et al., *Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome.* Proc Jpn Acad Ser B Phys Biol Sci, 2009. **85**(8): p. 348-62.
13. Stadtfeld, M., et al., *Induced pluripotent stem cells generated without viral integration.* Science, 2008. **322**(5903): p. 945-9.
14. Zhou, H., et al., *Generation of induced pluripotent stem cells using recombinant proteins.* Cell Stem Cell, 2009. **4**(5): p. 381-4.
15. Huangfu, D., et al., *Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds.* Nat Biotechnol, 2008. **26**(7): p. 795-7.
16. Judson, R.L., et al., *Embryonic stem cell-specific microRNAs promote induced pluripotency.* Nat Biotechnol, 2009. **27**(5): p. 459-61.
17. Thomson, J.A., et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
18. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
19. Totonchi, M., et al., *Feeder- and serum-free establishment and expansion of human induced pluripotent stem cells.* Int J Dev Biol, 2010. **54**(5): p. 877-86.
20. Stover, A.E. and P.H. Schwartz, *Adaptation of human pluripotent stem cells to feeder-free conditions in chemically defined medium with enzymatic single-cell passaging.* Methods Mol Biol, 2011. **767**: p. 137-46.
21. Ausubel, L.J., P.M. Lopez, and L.A. Couture, *GMP scale-up and banking of pluripotent stem cells for cellular therapy applications.* Methods Mol Biol, 2011. **767**: p. 147-59.
22. Chen, G., et al., *Chemically defined conditions for human iPSC derivation and culture.* Nat Methods, 2011. **8**(5): p. 424-9.
23. Miyazaki, T., et al., *Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells.* Nat Commun, 2012. **3**: p. 1236.
24. Mei, Y., et al., *Combinatorial development of biomaterials for clonal growth of human pluripotent stem cells.* Nat Mater, 2010. **9**(9): p. 768-78.
25. Lu, H.F., et al., *A 3D microfibrous scaffold for long-term human pluripotent stem cell self-renewal under chemically defined conditions.* Biomaterials, 2012. **33**(8): p. 2419-30.
26. Draper, J.S., et al., *Culture and characterization of human embryonic stem cells.* Stem Cells Dev, 2004. **13**(4): p. 325-36.
27. Ludwig, T.E., et al., *Derivation of human embryonic stem cells in defined conditions.* Nat Biotechnol, 2006. **24**(2): p. 185-7.
28. Meng, G., S. Liu, and D.E. Rancourt, *Synergistic effect of medium, matrix, and exogenous factors on the adhesion and growth of human pluripotent stem cells under defined, xeno-free conditions.* Stem Cells Dev, 2012. **21**(11): p. 2036-48.

29. Puri, M.C. and A. Nagy, *Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on.* Stem Cells, 2012. **30**(1): p. 10-4.
30. Bilic, J. and J.C. Izpisua Belmonte, *Concise review: Induced pluripotent stem cells versus embryonic stem cells: close enough or yet too far apart?* Stem Cells, 2012. **30**(1): p. 33-41.
31. Mikkelsen, T.S., et al., *Dissecting direct reprogramming through integrative genomic analysis.* Nature, 2008. **454**(7200): p. 49-55.
32. Wernig, M., et al., *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state.* Nature, 2007. **448**(7151): p. 318-24.
33. Stadtfeld, M. and K. Hochedlinger, *Induced pluripotency: history, mechanisms, and applications.* Genes Dev, 2010. **24**(20): p. 2239-63.
34. Deng, J., et al., *Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming.* Nat Biotechnol, 2009. **27**(4): p. 353-60.
35. Doi, A., et al., *Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts.* Nat Genet, 2009. **41**(12): p. 1350-3.
36. Bock, C., et al., *Reference Maps of human ES and iPS cell variation enable high-throughput characterization of pluripotent cell lines.* Cell, 2011. **144**(3): p. 439-52.
37. Yamanaka, S., *Induced pluripotent stem cells: past, present, and future.* Cell Stem Cell, 2012. **10**(6): p. 678-84.
38. Hu, B.Y., et al., *Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency.* Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4335-40.
39. Zhang, J., et al., *Functional cardiomyocytes derived from human induced pluripotent stem cells.* Circ Res, 2009. **104**(4): p. e30-41.
40. Mauritz, C., et al., *Generation of functional murine cardiac myocytes from induced pluripotent stem cells.* Circulation, 2008. **118**(5): p. 507-17.
41. Liu, Z., et al., *Current status of induced pluripotent stem cells in cardiac tissue regeneration and engineering.* Regen Med Res, 2013. **1**(1): p. 6.
42. Cho, G.S., L. Fernandez, and C. Kwon, *Regenerative medicine for the heart: perspectives on stem-cell therapy.* Antioxid Redox Signal, 2014. **21**(14): p. 2018-31.
43. Osafune, K., et al., *Marked differences in differentiation propensity among human embryonic stem cell lines.* Nat Biotechnol, 2008. **26**(3): p. 313-5.
44. Shi, Y., et al., *Induced pluripotent stem cell technology: a decade of progress.* Nat Rev Drug Discov, 2017. **16**(2): p. 115-130.
45. *www.ClinicalTrials.gov.*
46. Mandai, M., et al., *Autologous Induced Stem-Cell-Derived Retinal Cells for Macular Degeneration.* N Engl J Med, 2017. **376**(11): p. 1038-1046.
47. Kimbrel, E.A. and R. Lanza, *Current status of pluripotent stem cells: moving the first therapies to the clinic.* Nat Rev Drug Discov, 2015. **14**(10): p. 681-92.
48. Trounson, A. and N.D. DeWitt, *Pluripotent stem cells progressing to the clinic.* Nat Rev Mol Cell Biol, 2016. **17**(3): p. 194-200.
49. Lee, A.S., et al., *Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies.* Nat Med, 2013. **19**(8): p. 998-1004.
50. Lund, R.J., E. Narva, and R. Lahesmaa, *Genetic and epigenetic stability of human pluripotent stem cells.* Nat Rev Genet, 2012. **13**(10): p. 732-44.
51. Liu, Z., et al., *The tumourigenicity of iPS cells and their differentiated derivates.* J Cell Mol Med, 2013. **17**(6): p. 782-91.
52. Miura, K., et al., *Variation in the safety of induced pluripotent stem cell lines.* Nat Biotechnol, 2009. **27**(8): p. 743-5.
53. Hanna, J., et al., *Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin.* Science, 2007. **318**(5858): p. 1920-3.
54. Pearl, J.I., et al., *Pluripotent stem cells: immune to the immune system?* Sci Transl Med, 2012. **4**(164): p. 164ps25.
55. de Almeida, P.E., et al., *Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance.* Nat Commun, 2014. **5**: p. 3903.
56. Huang, K., et al., *Neural progenitor cells from human induced pluripotent stem cells generated less autogenous immune response.* Sci China Life Sci, 2014. **57**(2): p. 162-70.
57. Raya, A., et al., *Disease-corrected haematopoietic progenitors from Fanconi anaemia induced pluripotent stem cells.* Nature, 2009. **460**(7251): p. 53-9.
58. Park, I.H., et al., *Disease-specific induced pluripotent stem cells.* Cell, 2008. **134**(5): p. 877-86.
59. Ye, L., et al., *Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases.* Proc Natl Acad Sci U S A, 2009. **106**(24): p. 9826-30.

60. Dimos, J.T., et al., *Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons.* Science, 2008. **321**(5893): p. 1218-21.
61. Pedrosa, E., et al., *Development of patient-specific neurons in schizophrenia using induced pluripotent stem cells.* J Neurogenet, 2011. **25**(3): p. 88-103.
62. Kazuki, Y., et al., *Complete genetic correction of ips cells from Duchenne muscular dystrophy.* Mol Ther, 2010. **18**(2): p. 386-93.
63. Li, Z., et al., *Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells.* Stem Cells Dev, 2011. **20**(10): p. 1701-10.
64. Zhang, J., et al., *A human iPSC model of Hutchinson Gilford Progeria reveals vascular smooth muscle and mesenchymal stem cell defects.* Cell Stem Cell, 2011. **8**(1): p. 31-45.
65. Narsinh, K.H., et al., *Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells.* J Clin Invest, 2011. **121**(3): p. 1217-21.
66. Qian, X., et al., *Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure.* Cell, 2016. **165**(5): p. 1238-54.
67. Desnuelle, C., et al., *A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis. ALS riluzole-tocopherol Study Group.* Amyotroph Lateral Scler Other Motor Neuron Disord, 2001. **2**(1): p. 9-18.

Personal contributions to the published works of this thesis (Melanie Generali)

Chapter 1: Dijkman P.E., Generali M, et al.

Encyclopedia of Biomedical Polymers and Polymeric Biomaterials, 2015
(Article category: *book chapter*, Copyright: 9781439898796)

Writing of chapters: Cell Sources for Cardiovascular Tissue Engineering; Polymeric starter matrices for cardiovascular tissue engineering; Vascular Tissue Engineering; creating Table 1

Chapter 2: Generali M., et al.

Colloid and Surfaces B: Biointerfaces (under revision)
(Article category: *original research article*)

Experimental contributions (Generali M. only):
Phenotyping of human fibroblasts; Fabrication of composite PGA-P4HB, PGA-PLA, and PGA-PCL patches; histological analysis (H&E, Masson trichome, and polarization microscopy); Biomechanical analysis of tissue-engineered patches

Experimental contributions (Generali M. together with co-authors):
Surface morphology of biomaterials (scanning electron microscopy); Quantitative Tissue analyses (ECM)

Non-experimental contributions (Generali M. only):
Study design and planning of study; writing of entire manuscript; submission and correspondence with editorial office; all revisions (R1)

Experimental steps contained in the published work – without personal contribution of Generali M.
Isolation of human fibroblasts and proliferation assay

Chapter 3: Generali M., et al.

Npj Nature Regenerative Medicine (Submitted)
(Article category: *original research article*)

Experimental contributions (Generali M. only):
Isolation and reprogramming of PBMC; virus production and concentration; characterization of PBMC derived iPSCs including quantitative RT-PCR, alkaline phosphatase assay, immunofluorescence of pluripotency markers; in-vitro embryoid body formation and analysis; differentiation and characterization of smooth muscle cells and endothelial cells derived from hiPSCs (quantitative RT-PCR and immunofluorescence for specific markers); fabrication of scaffolds for TEVG; qualitative (histological analysis:H&E, Masson Trichome, and polarization microscopy) and quantitative (ECM) analysis of TEVGs; development of a bioreactor for small diameter TEVGs

Experimental contributions (Generali M. together with co-authors):
Cell seeding of PGA-P4HB derived TEVGs, teratoma assay and animal care, stochastical analysis

Non-experimental contributions (Generali M. only):
Study design and planning of study; writing of entire manuscript; submission and correspondence with editorial office; drafting and submission of all underlying ethical permissions [KEK-ZH-2014-0430; ZH-171/2014];

Experimental steps contained in the published work – without personal contribution of Generali M.
None

Curriculum Vitae

KNOWLEDGE OF

Research methods
Preparing documentation
Verifying data
Report writing
Designing databases
Data interrogation
Data entry
Statistical analysis
Data mining
Coordinating studies

PERSONAL SKILLS

Resourceful
Detailed & accurate
Enthusiastic
Practical & dexterous
Patient
Organized
Detail orientated

Melanie Generali

PROFESSIONAL EXPERIENCE

Institute for Regenerative Medicine, University of Zurich, Switzerland
PhD candidate 1/2014 to present

- Outlining and planning research project objectives and scheduling activities independently
- Keeping senior managers and researchers updated
- Establishing and maintaining collaborations
- Initiator and executer of ethical applications
- Training and teaching of students as a teaching assistant of the physiology class over two years
- Involved in the recruitment and training of new staff
- Experience of applying for research funding Forschungskredit Candoc 60`000CHF
- Writing scientific publications/presenting data
- Building effective working relationships with both work colleagues and clients

The Institute of Cancer Research Sutton, United Kingdom
Research Assistant 6/2013 – 12/2013

- Responsible for project progress documentation and schedule updates
- Designing and conducting questionnaire surveys
- Collecting and managing research data
- Acquiring scientific technics/methods and establishing new protocols

University Children`s Hospital, Cancer Research Zurich, Switzerland
Master Student 9/2011 – 05/2013

- Keeping accurate records of experiments and investigations
- Finding relevant information quickly and efficiently
- Helping to prepare reports and conclusions for publication
- Updating and maintaining logs and filing systems

Leibniz Institute for Science and Mathematics Education, Kiel, Germany
Research assistant 2/2009 – 7/2011

- Customer service
- Handling daily business (homepage design, data management)
- Event management of conferences

ACADEMIC QUALIFICATIONS

1/2014 to present	PhD Program in Integrative Molecular Medicine, Life Science Zurich Graduate School, Switzerland
9/2011 – 5/2013	M.Sc. Master of Science in Biology, University of Zurich, Switzerland
10/2008 – 8/2011	B.Sc. Bachelor of Science in Biology, Christian-Albrechts-University of Kiel, Germany

PROFESSIONAL TRAINING

5/2016-6/2017	GCP Modules 1, 2 & 3, Clinical Trial Center, Zurich
3/2014-10/2015	Supervision of physiology lab classes for medical students, Zurich Center for Integrative Human Physiology, University of Zurich
10/2014	Training on “Project Management for Research”, University of Zurich
10/2014	Comprehensive course in flow cytometry, University of Zurich
6/2014	Introductory Course in Laboratory Animal Science, accredited as FELASA Category B Course, University of Zurich

LANGUAGES

German	Native Speaker
English	Working language C2
Spanish	Basic
Russian	Basic

COMPUTER SKILLS

MS Office	Working
Graph Pad Prism	Working
Adobe Photoshop	Introduction and advanced course

BIBLIOGRAPHY

Original peer-reviewed publication (first author)	2 accepted, 1 s
Original peer-reviewed publication (co author)	1 published, 2 submitted
Peer-reviewed review articles/ book chapter	2 published
Public presentation	3 presented
Poster presentation	2 presented

ACTIVITIES AND INTERESTS

Painting and photographing	Exhibition of my paintings/photos
Travelling	Interest in South and Middle America, Publishing travel report
Sport	Hiking, Swimming

Peer reviewed publications

Generali, Melanie*; Kehl, Debora*; Capulli, Andrew K.; Parker, Kevin K.; Hoerstrup, Simon P.*; Weber, Benedikt*. Comparative Analysis of Poly-glycolic Acid-based Hybrid Polymer Starter Matrices for In vitro Tissue Engineering, Colloid and Surfaces B:Biointerfaces, 2017

Kehl, Debora*; **Generali, Melanie***; Görtz, Sabrina; Geering, Diego; Slamecka, Jaroslav; Hoerstrup, Simon P.; Bleul, Ulrich; Weber, Benedikt. Amniotic fluid cells show higher pluripotency-related gene expression than allcantoic cells, Stem Cells and Development, 2017

Böttner, Martina; Fricke, Tobias; **Müller**, **Melanie;** Barrenschee, Martina; Hellwig, Ines; Deuschl, Günther; Schneider, Susanne; Egberts, Jan-Hendrik; Becker, Thomas; Fritscher-Ravens, Annette; Arlt, Alexander; Wedel, Thilo. Alpha-synuclein is associated with the synaptic vesicle apparatus in the human and rat enteric nervous system, Brain Research, 2015

Generali, Melanie; Casanova, Elisa A., Kehl, Debora; Bode, Peter K.; Hoerstrup, Simon P.; Cinelli, Paolo*, Weber, Benedikt*; Endothelialized bioengineered vascular grafts based on peripheral blood mononuclear cell-derived induced pluripotent stem cells, *[submitted]*

Milton, Christopher; Selfe, Joanna; Missiaglia, Edoardo; Aladowicz, Ewa; Walters, Zoë S.; Gatz, Susanne A.; **Generali, Melanie**; Box, Gary; Velateni, Melanie; de Haven-Brandon, Alexis; Galiwango, David; Hayes, Angela; Clarke, Matthew; Izquierdo, Elisa; Gonzalez De Castro, David; Raynaud, Florence; Eccles, Suzanne; Shipley, Janet M. Preclinical efficacy of the FGFR inhibitor NVP-BGJ398 in fusion positive rhabdomyosarcomas is associated with dependency on FGFR2 to maintain cell growth, *[submitted]*

Casanova, Elisa A., **Generali, Melanie;** Satheesha, Sampoorna; Bode, Peter K.; Cinelli, Paolo; Schäfer, Beat W. PAX3-FOXO1 increases fibroblast reprogramming efficiency and drives selfrenewal in alveolar rhabdomyosarcoma, *[submitted]*

Book chapter

Dijkman, Petra E*; **Generali, Melanie***; Weber, Benedikt; Hoerstrup, Simon P. Cardiovascular Tissue Engineering, Polymeric Starter Matrices for. In *Encyclopedia of Biomedical Polymers and Polymeric Biomaterials*; Mishra, M. Ed.; Taylor and Francis: New York, 2015.

Review

Generali, Melanie*, Dijkman, Petra E.*, Hoerstrup, Simon P. Bioresorbable Scaffolds For Cardiovascular Tissue Engineering. In *EMJ Int Cardiol;* 2014.

Public presentation

Reprogramming of peripheral blood mononuclear cells into induced pluripotent stem cells for the in-vitro manufacture of bioengineered vascular constructs, The Biology of Regenerative Medicine, Hinxton, Cambridge, UK, April 2017

Reprogramming of peripheral blood mononuclear cells into induced pluripotent stem cells for the in-vitro manufacture of bioengineered vascular constructs, 13th Winter Brain & Heart Symposium 2017, Sils-Maria, Switzerland, March 2017

Reprogramming of peripheral blood mononuclear cells into induced pluripotent stem cells for the in-vitro manufacture of bioengineered vascular constructs, Retreat of the program of Integrative Molecular Medicine, Magglingen, Switzerland, June 2016

Poster presentation

Reprogramming of peripheral blood mononuclear cells into induced pluripotent stem cells for the in-vitro manufacture of bioengineered vascular constructs, Advances of Stem Cells and Regenerative Medicine, Heidelberg, Germany, May 2017

Reprogramming of peripheral blood mononuclear cells into induced pluripotent stem cells for the in-vitro manufacture of bioengineered vascular constructs, The Biology of Regenerative Medicine, Hinxton, Cambridge, UK, April 2017

Acknowledgements

While my name may be alone on the front cover of this thesis, I am by no means its sole contributor. Rather, there are a number of people behind this work who deserve to be both acknowledged and thanked here.

First, I would like to thank Prof. Dr. Dr. **Simon P. Hoerstrup** for giving me the opportunity to pursue my Ph.D. thesis in his laboratory. I would like to express my appreciation to my supervisor PD Dr. Dr. **Benedikt Weber**. Thank you for giving me the opportunity to participate in different projects and helping me become an independent researcher.

Furthermore, I would like to express my special appreciation to PD Dr. **Paolo Cinelli**. Encouraging my need to develop and follow many ideas, his advice, foresight and interesting discussions both about science and otherwise, and having enough faith in me and my abilities to direct the project. The atmosphere was always open, friendly, informative and collaborative and I am indebted to him for training me to be a confident researcher.

I would also like to thank my committee members, Prof. Dr. **François Verrey**, Prof. Dr. **Arnold von Eckardstein**, and Prof. Dr. **Benjamin Gantenbein**. Thank you for the lively discussions we had and the thoughtful input you gave on my projects.

Additionally, I would like to thank all members of the Institute of Regenerative Medicine at Moussonstrasse 13. I had a great time! **Debora**, **Laura**, **Marko**, and **Ursi** deserve special thanks for their friendship. They made my time here so special.
A huge thank you goes also to my second lab family (the team of the division of trauma surgery). I am grateful to **Elisa** for always taking the time to discuss research and and other things. Thank you to **Daisy**, **Urs**, **Sonja^2**, and **Benjamin** for sharing cake, time and good mood.

Finally, I would like to thank the most important persons in my life, my family and my friends.

Trish, ich kenne kaum eine nerdigere Wissenschaftlerin. Danke für deine Freundschaft, Ratschläge und verrückten Ideen.

Meiner Familie, insbesondere meiner **Mama**, möchte ich für die Unterstützung und den Glauben an mich danken. Der Nobelpreis ist nahe ;). Ich werde immer wieder gerne nach Hause kommen. Ich hab dich lieb.

Roberto, dir danke ich besonders. Du bist mein Ehemann, bester Freund, Vater unserer (bald) Kinder und Liebe meines Lebens. In den letzten Jahren hast du mich immer unterstützt und ermutigt, egal was war. Zellen zu zählen ist nicht deine Stärke, aber ich liebe dich trotzdem. ;)
Auch dir danke ich, **Robertino**, du hast so schön geschlafen wenn wir im Labor waren und hilfst Mama so viel und oft!